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PULMONARY

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#959 - ALTWICKER

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1974

Grant application No. 959

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Gardner, Liebow and Wyatt

Subject: Elmar R. Altwicker, Ph.D., Rensselaer Polytechnic Institute
New application No. 959
"The Effects of Pollutant Gases, Particles, and Industrial
Atmospheres on Lung Surfactant"

History

During the summer of 1973 we received a "presentation" from this investigator via Philip Morris Incorporated. As the proposal seemed clearly within CTR program scope, we provided application forms.

Request

Application No. 959 requests \$78,640 plus two additional years.

Documents Submitted (attached)

1. Application, undated, received January 28, 1974 (28 pages)
2. Biographical information on Drs. Altwicker and Balint

FWN:gh

Encls.


F.W.N.

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RPI Proposal No. 117(74R)B101(11)

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

(212) 421-8385

JAN 28 1974

Application for Research Grant

Date:

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Elmar R. Altwicker, Associate Professor, Ph.D.

2. Institution & address:

RENSSELAER POLYTECHNIC INSTITUTE
Troy, New York 12181

3. Department(s) where research will be done or collaboration provided:

Division of Bio-Environmental Engineering, Rensselaer Polytechnic Institute
Division of Gastroenterology, Albany Medical College

4. Short title of study:

THE EFFECTS OF POLLUTANT GASES, PARTICLES, AND INDUSTRIAL ATMOSPHERES ON
LUNG SURFACTANT

5. Proposed starting date: 1 July 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: cf. Page 12 of the attached proposal

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8. Brief statement of working hypothesis:

Turnover rates of lung surfactant increase when laboratory animals are subjected to tobacco smoke. Since different portions of the surfactant moiety appear to turn over at different rates, long term studies are proposed in which the decay of radioactive isotopes is followed in both the lecithin and water soluble precursor portions in order to clarify the mechanism. If long term turnover studies using pollutants other than tobacco smoke are combined with determination of composition, the results may give further clues as to the possibility that the surfactant "guards" the air-lung surface interface. Experiments with known particle size fractions are included. In conjunction with a study of EFA-deficient animals the studies are aimed at the question of possible chronic lung damage related to higher turnover rates and the possibility of depletion of the surfactant leading to pulmonary edema. (cf. Page 10-12).

9. Details of experimental design and procedures (append extra pages as necessary)

cf. Pages 13-20 of the attached proposal.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

cf. Section E, Page 22, of the attached proposal

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

cf. Section G, Page 29, of the attached proposal

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Same as 12.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Principal Investigator (Dr. E. R. Altwicker)

20%A*

\$ 3,600

50%S*

2,040

Postdoctoral Fellow (Dr. J. Burnell)

100%C*

10,000

Technical

Graduate Assistant (to be recruited)

50%A

4,780

Graduate Assistant (to be recruited)

50%A

4,780

Graduate Assistant (to be recruited)

100%SM*

1,665

Technician (to be recruited)

100%C

7,800

Technician (to be recruited)

100%C

7,800

*A-Academic Year; S-2 Summer Months

C-Calendar Year; SM-3 Summer Months

Sub-Total for A \$42,465

B. Consumable supplies (by major categories)

Rats (purchase and care)

1,500

Stable and Radioactive Isotopes

5,000

Calibration Gases, Glassware, Solvents

Chemicals, Filters

4,500

Sub-Total for B \$11,000

C. Other expenses (itemize)

Equipment Maintenance

300

Domestic Travel to Scientific Meetings

500

Computer Usage

2,500

Purchase Order with Albany Medical

College for the services of Dr. J.A. Balint:

Salaries-10% time, calendar year -\$4,000

6,400

Indirect Cost-60% of Salaries - 2,400

\$ 9,700

Total \$6,400

Sub-Total for C

Running Total of A + B + C

\$63,165

D. Permanent equipment (itemize)

Chemiluminescent Ozone Monitor
and Recorder

6,000

Sub-Total for D 6,000E 9,475Total request \$78,640

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | 44,590 | 6,000 | 9,700 | --- | 9,044 | \$69,334 |
| Year 3 | 46,820 | 6,000 | 9,000 | --- | 9,273 | \$71,093 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--------------------------------|----------|--------------------|
| Acute & Chronic Effects of Tobacco Smoke (JAB) | AMA-ERI | \$57,126 | 5/1/71 - 8/31/75 |
| Studies on the Metabolism of Pulmonary Surfactant (JAB-ERA) | NIH HL 15273 | 167,865 | 9/1/72 - 8/31/75 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--|----------|--------------------|
| Plasma, Erythrocyte and Biliary Lipids During Cheno Deoxycholate Therapy (JAB) | NIH | \$66,450 | 7/1/74 - 6/30/77 |
| Atmospheric Sulfur and Its Role in Aquatic Cycling (ERA) | EPA | 98,000 | 2/1/74 - 8/31/75 |
| Mechanism of the Inhibition of Sulfur Dioxide Oxidation During Wet Scrubbing (ERA) | NSF | 46,100 | 5/1/74 - 4/30/76 |
| An Appraisal of Energy Alternatives Available to N.T. State (ERA), M. Becker, Director | ASDA (Atomic Space and Development Adm., N.Y. State) | 70,000 | 12/1/73 - 8/31/74 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Elmar R. AltwickSignature Elmar R. Altwick Date 1/17/74Telephone (518) 270 - 6554
Area Code Number Extension

Checks payable to

Rensselaer Polytechnic Institute

Mailing address for checks

Troy, New York 12181

Responsible officer of institution

Typed Name H. C. MatticeTitle Director, Office of Contracts and GrantsSignature H. C. Mattice Date 1/23/74Telephone (518) 270 - 6281
Area Code Number Extension

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RENSSELAER POLYTECHNIC INSTITUTE
Troy, New York 12181

RPI Proposal No. 117(74R)B101(11)

entitled

THE EFFECTS OF POLLUTANT GASES, PARTICLES AND
INDUSTRIAL ATMOSPHERES ON LUNG SURFACTANT

Submitted on behalf of

Elmar R. Altwicker
Associate Professor
Division of Bio-Environmental Engineering
Rensselaer Polytechnic Institute
Troy, New York 12181

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January 1974

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A. INTRODUCTION

1. Objectives

Many investigations have been undertaken to elucidate the biosynthetic pathways of surfactant lecithin production and to clarify the in vivo assembly of the surfactant system. This system is currently thought to consist of phospholipid, predominantly dipalmitoyl lecithin, and protein. It has been suggested that these are assembled within Type II alveolar cells and secreted and catabolyzed as a unit. The evidence is chiefly indirect, however.

The surfactant is produced in the lung and is essential to its normal function; the surfactant prevents collapse of air spaces at physiological pressures. Interference with production and subsequent activity of surfactant could lead to higher surface tension. As a result it will require greater pressure to inflate the lung so that pulmonary compliance will be reduced. Interference with surfactant activity leads to abnormality of lung function (respiratory distress syndrome).

It has been speculated that during episodes of high air pollution or exposure to toxic gases there may be a short-lived interference with surfactant activity. Absence of a permanent reduction of surfactant production are primarily based on surface tension measurements, which are based on questionable rationale.

The purpose of the studies described below is to use single pollutant inhalation, synergistic mixtures, industrial atmospheres, and highly polluted atmospheres and dietary manipulation to produce changes in synthetic and secretory rates and to monitor concentration and composition of surfactant to further elucidate basic mechanisms of surfactant activity and metabolism.

2. Background

Under a joint NIH-grant the two principal investigators are currently engaged in a study of the biosynthesis of the surfactant system in vivo and in vitro in order to learn more about relative importance of different pathways, and a study of the effect of tobacco smoke and tobacco smoke particulate fractions on surfactant turnover rates.

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One of us (JAB) and his colleagues at AMC have published a number of papers concerned with phospholipid metabolism in normal and EFA deficient rats and hamsters especially with reference to hepatic and biliary lecithin metabolism (1-6). These studies have demonstrated the existence of several functionally distinct pools of lecithin within the liver (1,2). Furthermore, it was shown that the synthesis, as well as secretion, of biliary lecithin was accelerated by increasing bile salt loads (5). In addition, these studies demonstrated that certain types of lecithin were preferentially synthesized by either the direct Kennedy pathway or the methylation pathway of Greenberg and Bremer (2,4). Also published have been studies on the methodology of phospholipid analysis by column or argentation chromatography (7,8) as well as studies on the neutral fraction and phospholipids in a number of lipid storage diseases (9,10,11).

During the past three years we have been engaged in studies in the metabolism of phospholipids in different tissues of dogs and rats and of the possible effects of exposure to tobacco smoke on these processes. The results of studies in dogs have recently been published (12). The data obtained in these experiments cast doubt on the importance of the methylation pathway to lecithin synthesis in lungs, as they showed no evidence of ^{14}C incorporation into lecithin following injection of methionine-methyl- ^{14}C or of ethanolamine-1,2- ^{14}C . The data also suggested decreased synthesis of pulmonary surfactant lecithin in dogs exposed to tobacco smoke for short periods of time (2-4 hours). These latter conclusions, however, are open to criticism, as they are based essentially on observations obtained after a single time period only.

More recently we have been engaged in studies of phospholipid metabolism in lungs, heart and liver of rats exposed to tobacco smoke for 2 - 3 days prior to isotope injection and of normal controls. These animals were then followed in groups of 4 for 2 - 48 hours. The data indicate statistically significant increase in the specific activity in surfactant lecithin of ^3H -glycerol ^{at} 2 hours ($p < .05$) (Fig. 1) in smoked as compared to control animals. Current experiments have

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³H-GLYCEROL - SURFACTANT

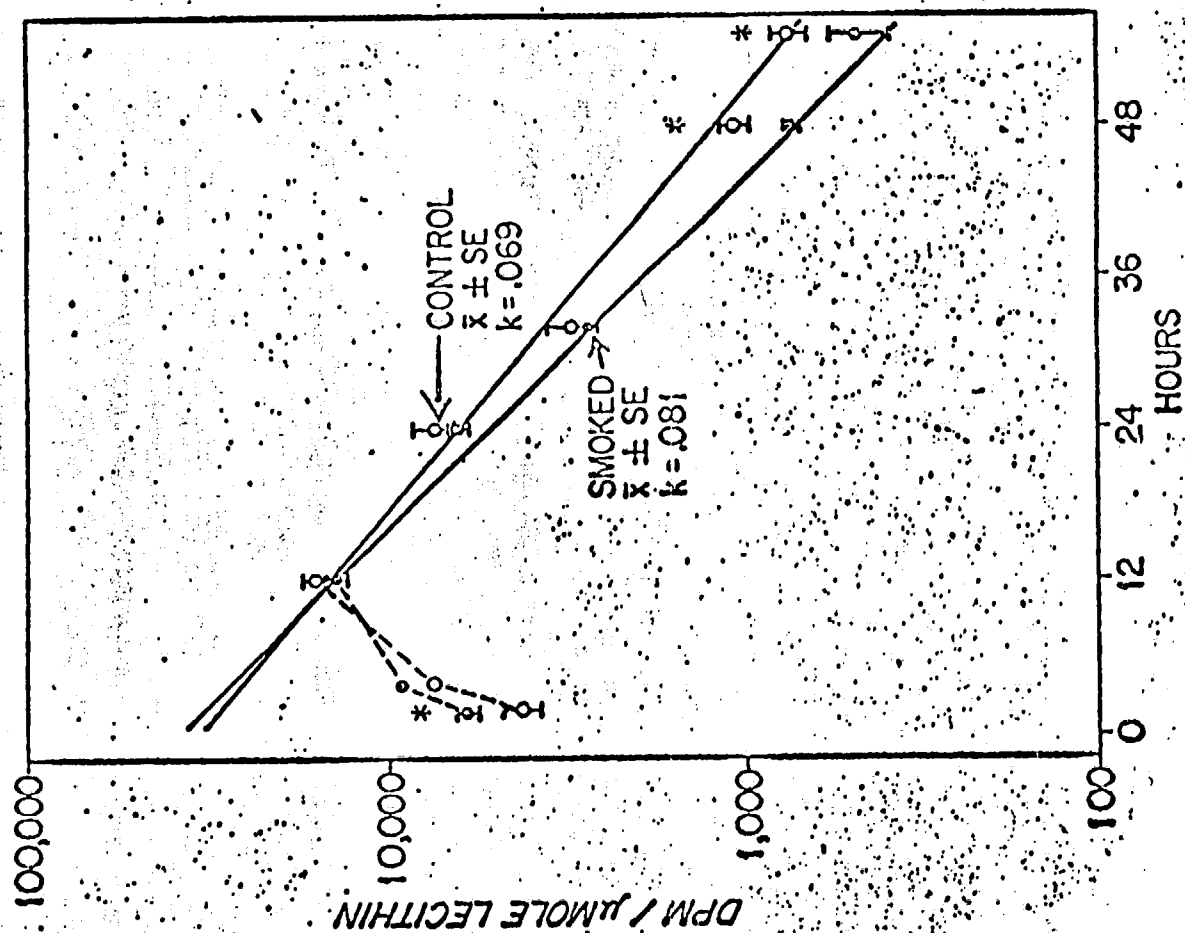


Figure 1

repeatedly confirmed this finding. This difference is still present though no longer significant at 4 hours, but is reversed at 12 hours. Glycerol- ^3H specific activity then declines more rapidly in surfactant lecithin from smoked animals than controls so that by 48 hours the difference is again significant ($p < .05$). Data for 32 hours and 60 hours support these findings. The slope for controls = .073 and for smoked animals = .083. Specific activity from choline-1,2- ^{14}C is higher in controls than in surfactant lecithin from smoked animals at 2 hours ($p < .05$) and remains higher throughout the 48 hours. (At 24 hours, $p < .05$ and 48 hours, $p < .01$). These results have now been fully confirmed in a second experiment. These data suggest increased turnover of surfactant lecithin in smoke exposed rats with perhaps reutilization of choline. Glycerol labeled in 2 position with ^3H is not reused as it is lost in the body water pool. More detailed studies for longer time periods and the use of selectively filtered smoke are currently under way. Data from these studies indicate that surfactant lecithin contains at least two pools, one with a half life of 12-15 hours, and the other with a half life of 50-52 hours in respect to decay of 2- ^3H -glycerol and 1- ^{14}C -palmitate labels.

These long term studies have shown, furthermore, that the turnover of glycerol and palmitate are similar as indicated, and shorter than that of choline-1,2- ^{14}C and ^{32}P phosphate. These latter tracers exhibit half lives of 20-25 hours and 70-80 hours for the rapid and slow compartments respectively. Possible reasons for this difference between glycerol and fatty acid on the one hand, and choline and phosphate on the other in lung is being investigated presently in in vitro studies of choline oxidation. It is of interest that no such differences can be demonstrated in liver lecithins.

The other investigator (ERA) and his students have been active in a number of areas in air pollution. Specifically, the effects of additives in fuel oils on the NO_x -formation during combustion has been studied using a small model combustor (13-17). In this connection, extensive investigations into measurements of NO and NO_2 have been made (Saltzman, PDSA, gas chromatography, and mass spectrometry have

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been used). Currently, all our measurements are done via chemiluminescence.

Another study has dealt with the kinetics of the ozone/olefin reaction (18) and the use of several measurement techniques for the detection of high and ambient ozone concentration (KI-method, olefin reaction, Mast ozone meter, chemiluminescent ozone monitor). Some studies have been carried out on the measurement of ozone in the presence of sulfur dioxide by these techniques.

Small particle measurements have been carried out in our laboratory by the use of Andersen heads on Hi-Vol samplers, the Lundgren Impactor, the Integrating Nephelometer, and the Condensation Nuclei Counter (CNC). Smoke filtration experiments using mass and CN-counts are currently in progress in connection with the surfactant turnover studies. In the past, a small study on ambient air pollutant measurements in a rural-recreational area (19) have been carried out. In this connection, benzo[a]pyrene was measured by fluorescence.

Another study - just commencing jointly with the Division of Air Resources of New York State Department of Environmental Conservation - is aimed at measurement and identification of ambient air hydrocarbons at a site in Troy, New York and in New York City.

3. Work Done by Others

Recognition of a surface active component of pulmonary edema fluid is very recent and derives from the observations of von Neergaard (20) and Pattle (21,22). Present knowledge concerning the metabolism, nature and disorders of the surfactant system have been extensively reviewed recently (23-25). Surfactant lecithin has been shown to be predominantly (50% or more) dipalmitoyl lecithin (26,27). Watkins (28) showed that this type of lecithin is particularly suitable for a spreading surfactant film due to its packing characteristics.

Morgan et al (26) demonstrated the presence of phosphatidyl dimethyl ethanolamine (PDME) in dog lung and lavage fluid (about 4-5% of total phospholipids). They showed that this compound was rich in palmitic acid and has surface active characteristics similar to those of dipalmitoyl lecithin. They suggested that

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PDME may be an intermediate in the synthesis of surfactant lecithin via the methylation pathway. Morgan (29) went on to demonstrate N-methyl transferase activity in dog lung microsomes. He showed that this enzyme in lung was most active with saturated PE as substrate and catalyzed the synthesis of saturated lecithin from mixed tissue PE. No such specificity was seen in the action of hepatic N-methyl-transferase. He further demonstrated that this enzyme activity was inhibited by high O_2 tensions. This enzyme activity was present in microsomes and in the lamellar bodies. Further evidence for the importance of the methylation pathway for the synthesis of dipalmitoyl lecithin of lung surfactant has come from studies of fetal lung lipid metabolism by Gluck and associates (30,31), Chida and Adams (32) and Morgan (33). These workers showed that just before birth synthesis of dipalmitoyl lecithin increased, together with increased activity of the methylation pathway and appearance PDME in lung extracts.

Other workers have questioned the predominant role of the methylation pathway in the biosynthesis of pulmonary surfactant. Thus, Spitzer et al (27, 34,35) have indicated that in vivo, using isotopically labeled choline, methionine-methyl and $^{32}PO_4$, the CDP-choline biosynthetic pathway is more important in adult female rats. These authors demonstrated very slow turnover of PDME relative to that of lecithin and could not confirm the similarity of fatty acid pattern between PDME and surfactant lecithin. Using similar techniques in dogs, we could not show significant incorporation of methyl group from methionine into pulmonary surfactant lecithin (12). Morgan (25,33) has recently concluded that the CDP-choline pathway may indeed be quantitatively more important under normal conditions. Of interest is a recent study by Pawlowski et al (36) which suggests the presence of a pool of surfactant type lecithin in lung parenchyma, associated with lamellar bodies, which may be the precursor or storage form of surfactant lecithin.

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Several investigators have suggested that the surfactant system contains a lipoprotein and that surfactant lecithin is excreted by the Type II cells in that form. Massaro (37) confirmed the presence of lamellar bodies in Type II alveolar cells (granular pneumocytes). He showed that in isolated cells from lung lavage fluid (50% of cells were Type II) labeled glucosamine, galactose and mannose was incorporated into protein in the microsomes and then transferred to the lamellar bodies shown to sediment at 15000 g (see also ref, 36). He suggested that this protein was part of the surfactant system. Massaro et al (38) confirmed these results both in vivo and in vitro using rats. They showed that epinephrin increased secretion of protein into the incubation medium in vitro whereas cyanide and cold (0°) inhibited this process. Pruitt et al (39) isolated a fraction from pig lung lavage fluid containing about 20% by weight of protein and rich in dipalmitoyl lecithin. They showed that this protein was immunologically not serum albumin. Similar composition of a fraction obtained from dog lung material has been reported (36). The significance of these data is thus questionable. The data of Spitzer and Norman (35) showing similar turnover of choline, phosphate and leucine in rat lung surfactant seem to support the concept that surfactant is a lipid-protein complex.

Recent studies by King and Clements (40) have demonstrated the presence of an immunologically distinct protein in lung lavage fluid, which by fluorescent microscopy, can be shown to be on the alveolar surface. This protein furthermore, is isolated from lung lavage material and lung homogenates, with the surface active saturated lecithin. These observations, therefore, present strong evidence to support the contention that surfactant is a lipoprotein.

Recent studies by Naimark (41) using labeled palmitic acid have indicated that alveolar macrophages may play an important role in the catabolism of surfactant lecithin. He showed that after injection of this tracer, radioactivity first appeared in lung DPL, then in surfactant lecithin and then in macrophages.

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However, studies on the turnover of surfactant lecithin are difficult to interpret, because of uncertainty as to which part of the molecule should be followed. Tierney et al (42) calculated a turnover of pulmonary surfactant to be 0.25 μ mole lecithin/gm of lung/hr from loss of compliance with rapid breathing. Using ^{14}C palmitate and U- ^{14}C -glucose (to label glycerol) they arrived at a turnover rate of 0.3 μ mole/gm/hr (42). They observed similar half-lives for pulmonary and surfactant lecithin (about 14 hours). Polyunsaturated lecithins turned over more slowly. Spitzer et al (34,35) obtained a half-life of surfactant lecithin using labeled choline and $^{32}\text{PO}_4$ of around 40 hours. Newman and Naimark (43) obtained a half life for surfactant lecithin of about 8-18 hours in normal animals and showed that hypoxia decreased turnover rate. Pilot studies in our laboratories (quoted above) gave a half-life for surfactant lecithin of 11.5 hours in normal animals, (8 hours in those exposed to tobacco smoke) using ^3H -glycerol. Half-life of choline-1,2- ^{14}C in the surfactant of these animals was 19-20 hours. These findings by different authors would be consistent with the concept of diglyceride exchange but clearly need further detailed evaluation.

Miller and Bondurant (44) showed that tobacco smoke blown over a surface film in a Wilhelmy trough lowered surface activity. Data to show that similar effects may occur in vivo are less convincing though evidence for such an effect in man has been presented (45). Fresolomo et al (46) suggested that surfactant lecithin is bound to protein by non-covalent bonding, thus accounting for its ready dissociation from protein. They considered that lecithin is probably present in liquid crystalline form stabilized by water, protein and counter ions. It is possible that these liquid crystals could thus be precipitated out by seeding with various particles in inspired air. Pulmonary artery occlusion and hypoxia, pulmonary edema and hyperbaric O_2 have been shown to reduce surfactant and lung compliance (for review see 25). Ladman and co-workers (47,48) have demonstrated changes in alveolar macrophages in cigarette smokers, with presence of large inclusions in the cells of the latter. Included in these inclusions were myelin

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figures, which might represent phagocytized surfactant lipoproteins. These findings are consistent with the metabolic studies cited above (41). Thickening of bronchiolar basement membrane has been reported in rats exposed to NO_2 for long periods (49). Similar changes have been reported after prolonged exposure to tobacco smoke (50). Recent work by Rhoades of long term exposure of rats to NO_2 gave evidence of altered surface activity and phospholipid composition of lung washings (51).

Gil (52) has drawn attention to the difficulties inherent to electron microscopic studies of lung tissue and surfactant. However, a number of studies (36,46,47,48) have recently shown that with proper attention to detail good preservation and images may be obtained. This technique has been used to correlate structure and lipid composition (53) and structure and metabolic function (36).

In terms of physico-chemical and photo-chemical interactions between surfactant and atmospheric or industrial pollutants dipalmitoyl lecithin (DPL) and egg lecithin (EL) monomolecular films and their interaction with NO_2 /olefin mixtures have been studied (54). Films of the saturated phospholipid (DPL) showed no interaction with any of the test atmospheres, while all NO_2 containing atmospheres effected a change with the unsaturated egg lecithin (EL); the evidence in the latter case was interpreted as a chemical reaction rather than a simple physical penetration phenomenon; contrast this with the findings of Rhoades (51). Since lung surfactant is predominantly DPL, it was speculated that the primary site of attack of pollutants were the materials of the capillary cell membranes rather than the surfactant. It is clear that isolated lung surfactant does contain some unsaturation, however (46,51); this should be susceptible to ozone attack. A recent symposium touched on many of the difficulties and conflicts in studying the interaction of pollutants with various loci within the lung (55). Coffin and his group (55) presented evidence to the effect that exposure to ozone had no significant effect on pulmonary surface activity or fatty acid composition of phospholipids. It was pointed out, however, that the surface tension studies were qualitative in nature

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and that a value of 15 dynes/cm reflected simply an arbitrary evaluation of the "presence" or "absence" of pulmonary surfactant and that quantitative inferences should not be made. In line with the in vitro findings cited above it was shown that at levels of exposure which produced extensive morphological changes in alveolar macrophages, there was no alteration in phospholipid composition or pulmonary surface activity and that alterations in cell structure are not mediated directly through a change in protective function by an agent such as DPL. Gardner et al (55) made similar observations in that the ability of DPL to protect macrophages was lost upon exposure to ozone, but the surface tension-lowering properties were unchanged. Experiments supposedly showing the destruction of surfactant by smog components have been reported (56).

Ramirez et al (55) studied diseased patients and found varying amounts of unsaturated fatty acids in the lipids of washings from such patients. Pattle (57) has speculated on the possible reaction of lung surfactant to nitrogen dioxide; however, cf. ref. 51.

An extensive body of literature exists on ozone and photochemical oxidant toxicology (58,59,60). Specifically, Goldstein (61) has suggested oxidation of fatty acids as a cause of ozone toxicity and pointed out the necessity for studies at ambient pollutant levels to determine whether lipid peroxidation can take place under such conditions in the lungs (62). Roehm (55) reported that oxidation of fatty acid methyl esters was increased by traces of nitrogen dioxide oxidations in films, and retarded both nitrogen dioxide and ozone oxidation in aqueous solutions. It has also been shown that a considerable portion of ozone is removed in the upper airways (63). Effects of surfaces on ozone makes it difficult to calculate the amount actually reaching the alveoli (64,65,66). In man, short-term ozone exposure has shown a significant increase in pulmonary resistance (64).

Changes in chemical composition of lung tissue homogenate, following ozone inhalation, could not be traced to a particular tissue component (67).

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Results on pulmonary deposition of aerosols have been reviewed (68).

Experimental and theoretical studies on the breathing of half-micron aerosols by Davies and co-workers have been interpreted as showing the complete absence of mechanical mixing in the alveolated airways (69).

4. Rationale

Current studies in our laboratories have shown that tobacco smoke inhalation accelerates the turnover of pulmonary and surfactant lecithin. Previous studies by others and ourselves show that the phosphoryl choline moiety of surfactant lecithin turns over more slowly than the glycerol and fatty acid portion. This could be explained by the diglyceride exchange postulated by Bjornstad & Bremer (70) or by assuming reutilization of phosphoryl choline. This question can be resolved by long term turnover studies in which the decay of radioactivity is followed in both the lecithin and its water soluble phosphorylated precursors. Long term turnover studies may in fact be the only way in which relationships between surfactant activity and permanent detrimental changes in the lung can be shown. There is evidence that chronic inhalation of irritant materials stimulates a large increase in the number of free alveolar phagocytes and decrease in the amount of extracellular surfactant in the alveoli (71).

The experiments with individual pollutants - some of which will be carried out shortly - will be aimed at the assessment of turnover rates to further substantiate evidence currently being gathered. However, it is unlikely that typical ambient concentrations of single pollutants will show permanent effects (51). It seems more plausible to concentrate on synergistic mixtures (O_3 , NO_2 ; O_3 , NO_2 , submicron particles; O_3 , SO_2 , trace metals in particles), highly polluted atmospheres, industrial atmospheres (which may be high in one or more toxic component, such as SO_2 , CO or Cl_2 , etc.), and special situations (i.e., H_2SO_4 mist which may arise to an increasing extent from automotive exhaust catalysts in 1975).

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Although it has been reported that DPL is not altered by ozone (cf. work done by others), this is perhaps somewhat surprising. Ozone does, of course, attack olefinic bonds faster than others, but it also attacks heteroatoms and C-H bonds. Moreover, attack on double bonds or heteroatoms may give rise to radicals which could form unstable hydroperoxides or to singlet oxygen (1O_2). Both types of reactions have been demonstrated in vitro (72,73,74). Pryor (74) has estimated that if all the ozone breathed by a human were converted to radicals, about 10^{-6} mole of radicals would be formed in the human body per day. Undoubtedly, only few of these radicals are involved in damaging processes but the suggestion that the lung mucus reacts with most of the ozone and protects lung tissue by a sacrificial mechanism lacks experimental substantiation and appears to be at variance with some in vivo experiments (cf. work done by others). If morphological changes occur while the material which presumably "guards" the air-lung surface interface remains intact, a sacrificial mechanism is not indicated (49). The turnover experiments and selective microscopic studies now in progress should help to clarify this point.

Certain synergistic combinations could be crucial as far as long term implications are concerned. Witness Selikoff's (75) findings on the synergistic effect of asbestos and cigarette smoke; it is expected that results from control experiments vs. toxic substances may soon be grossly predictable, based on the trend of our data; but it is considerably more difficult to predict which synergistic combinations of two or more substances may lead to higher turnover rates as well as chronic effects.

In addition, we have shown that in animals deficient in essential fatty acids (EFA) uptake of fatty acid by intestinal mucosa and of bile salts by liver is normal; however, removal of triglycerides into intestinal lymph (76) and secretion of bile salts and lecithin into hepatic bile is markedly impaired (6). These results, and similar data by other investigators (72), indicates an excretory defect in EFA deficient animals. It seems likely, therefore that a similar

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excretory defect may be produced in the lungs by EFA deficiency. Such an excretory block may so alter surfactant excretion as to produce more severe lung damage in animals exposed to pollutants. In liver EFA deficiency is associated with increased phospholipid turnover. If similar changes occur in lung, increased incorporation of isotope into intracellular lipids may be found. Simultaneous examination of protein synthesis using labeled amino acids under such circumstances could yield important information concerning the site of assembly of the surfactant system.

Since increased breathing rates can lead to increased turnover rates, this effect will have to be accounted for. Specific experiments to do this are outlined below.

Finally, human studies on turnover rates using stable isotopes are proposed.

Radioactive isotopes have limited usefulness for human studies, such as described above, because of the magnitude of the radiation dose involved in order to obtain reliable count rates. As it is clearly important to confirm animal data in man, we plan to develop the techniques for use of stable isotopes (deuterium and N^{15}) in human studies. We plan to use uniformly D_2 labeled palmitic acid from commercial sources. They are available, but very expensive at present. The biochemical and physiologic studies with stable isotopic tracers will be similar to those used with radioactive isotopes. The techniques, however, will need to be developed. Preliminary work in this approach has been started. (Dr. J. Burnell who recently completed her Ph.D. program at RPI, will join us for the specific purpose of developing these techniques in conjunction with Dr. John Hudson at RPI).

B. SPECIFIC AIMS

1. To obtain clarification of the sites and mode of biosynthesis and secretion and sites and modes of catabolism of surfactant in normal and EFA-deficient rats.

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2. To examine the effect of environmental pollutants, synergistic mixtures, highly polluted atmospheres, and typical industrial atmospheres upon these processes.
3. To study the effect these pollutant mixtures in an isolated heart-lung preparation (78).
4. To study certain substances (or groups of substances) from 2. in combination with cigarette smoke (and fractionated-as to particle size - smoke).
5. To study the effects of these pollutants upon cardiac and hepatic phospholipid metabolism, as controls for the above studies.
6. To study surfactant turnover rates in anesthetized and exercised rats (both controls) and those exposed to toxic substances in order to further delineate the reasons for the increased rates of catabolism.
7. To investigate surfactant turnover in humans using stable isotopes.
8. To develop computer models of the observed metabolic schemes, and thus to try to site of any metabolic effects observed.

C. METHODS OF PROCEDURE

Various groups of rats will be used for these studies and at least 8 animals in each group will be used to determine the mean value and SD for each time period. The groups of animals will be as follows:

1. Control - animals fed regular chow and not exposed to pollutants.
2. EFA deficient controls - weanling rats fed a fat free diet (Nutritional Biochemicals Company) supplemented with 4% tripalmitin for 12 weeks. Not exposed to pollutants.
3. Animals exposed to pollutants - fed regular chow.
4. EFA deficient animals fed as for 2 but exposed to pollutants for various time periods.
5. An additional control group will receive the fat free diet supplemented with 4% safflower oil, in order to rule out any effects due to difference in dietary carbohydrate content.

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During the isotopic studies animals are housed in special cages as illustrated below; future studies will be made using the modified inlet system shown.

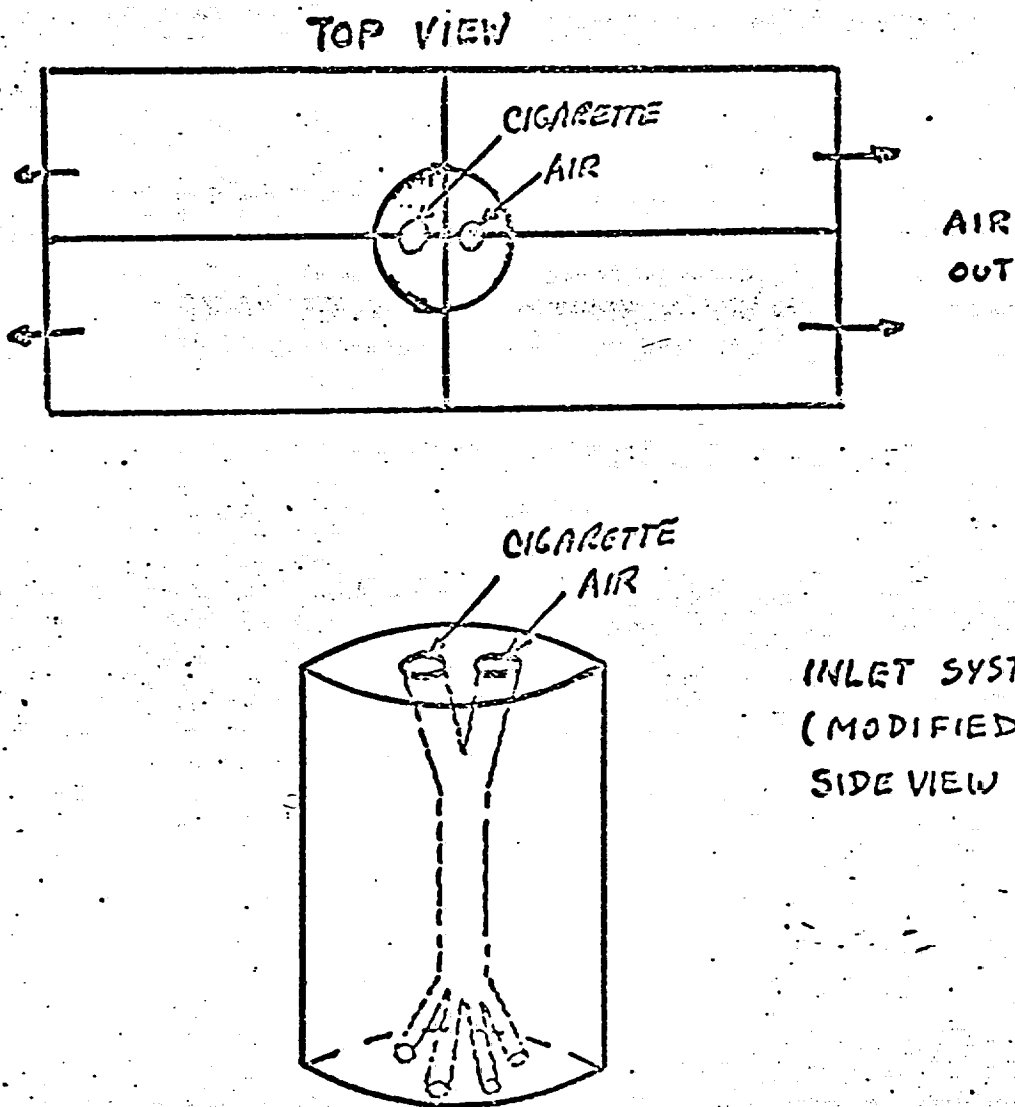


Figure 2

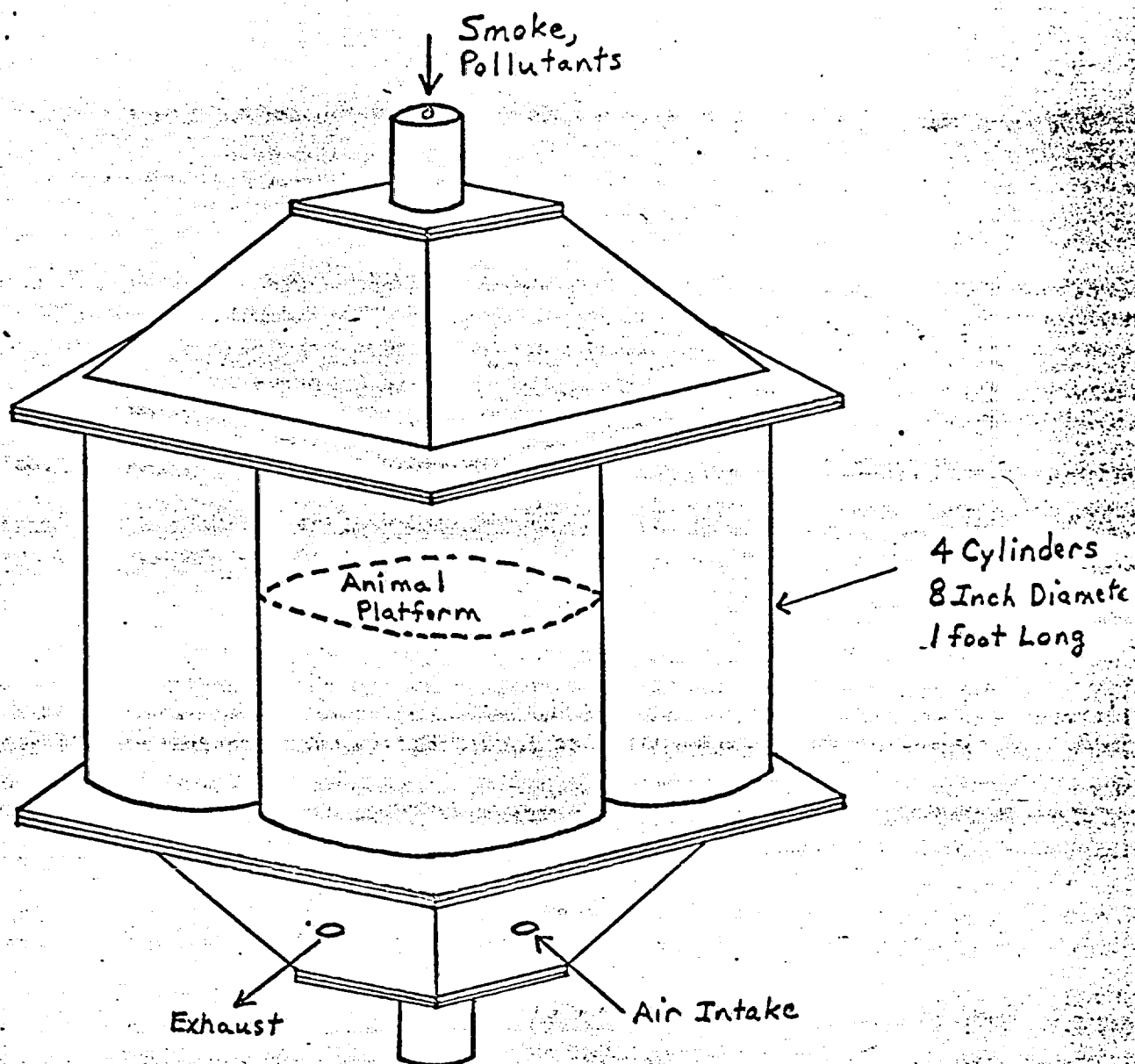
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Air is moved through the chamber by vacuum or a small pump and the two valves are electromagnetically operated through a timer, which allows room air or pollutant to enter the chamber in alternating cycles. When smoking the timing is adjusted to obtain a 30 cc puff at -30 cm H₂O pressure (2 secs each minute). In current runs, experimental animals are exposed to tobacco smoke from 20 unfiltered cigarettes per day for 3 days prior to isotope injection. They are continued to exposure at this rate to the time of sacrifice. Subsequent studies will be done with longer periods of exposure to tobacco smoke and other pollutants. In other experiments the intensity of exposure will be varied. The rats will be weighed before the start of exposure and daily thereafter and their food consumption recorded. If the experimental animals show significant differences in food intake from controls (non-pollutant exposed) additional pair fed control animals will be used.

Certain artifacts of the present chamber design which derive primarily from the large surface/volume ratio (which increases drastically when a rat is placed in the chamber) make it difficult to account for all losses and concentration gradients within the chamber. In the current studies this is especially true of condensation nuclei counts, for example, where the counter we are using has a high sampling rate (300 ml/min) and a relatively slow (~1 sec) response time.

A new chamber is under construction using currently recommended techniques (64,79), it will permit a variable S/V-ratio (Figs. 3 & 4). This chamber will be provided with suitable means of feeding and cleaning and concentration measurements for selected substances. It will permit a considerable variation in gas residence time (this is important for the study of periodic variations - diurnal, for example - as well as for the accounting of loss rates to the walls). Although we do not propose to construct a smog chamber some of the recent findings from smog chamber design and measurements are pertinent (80,81). Filtration techniques and efficiencies for smokes and aerosols have been discussed in detail (82,83,84).

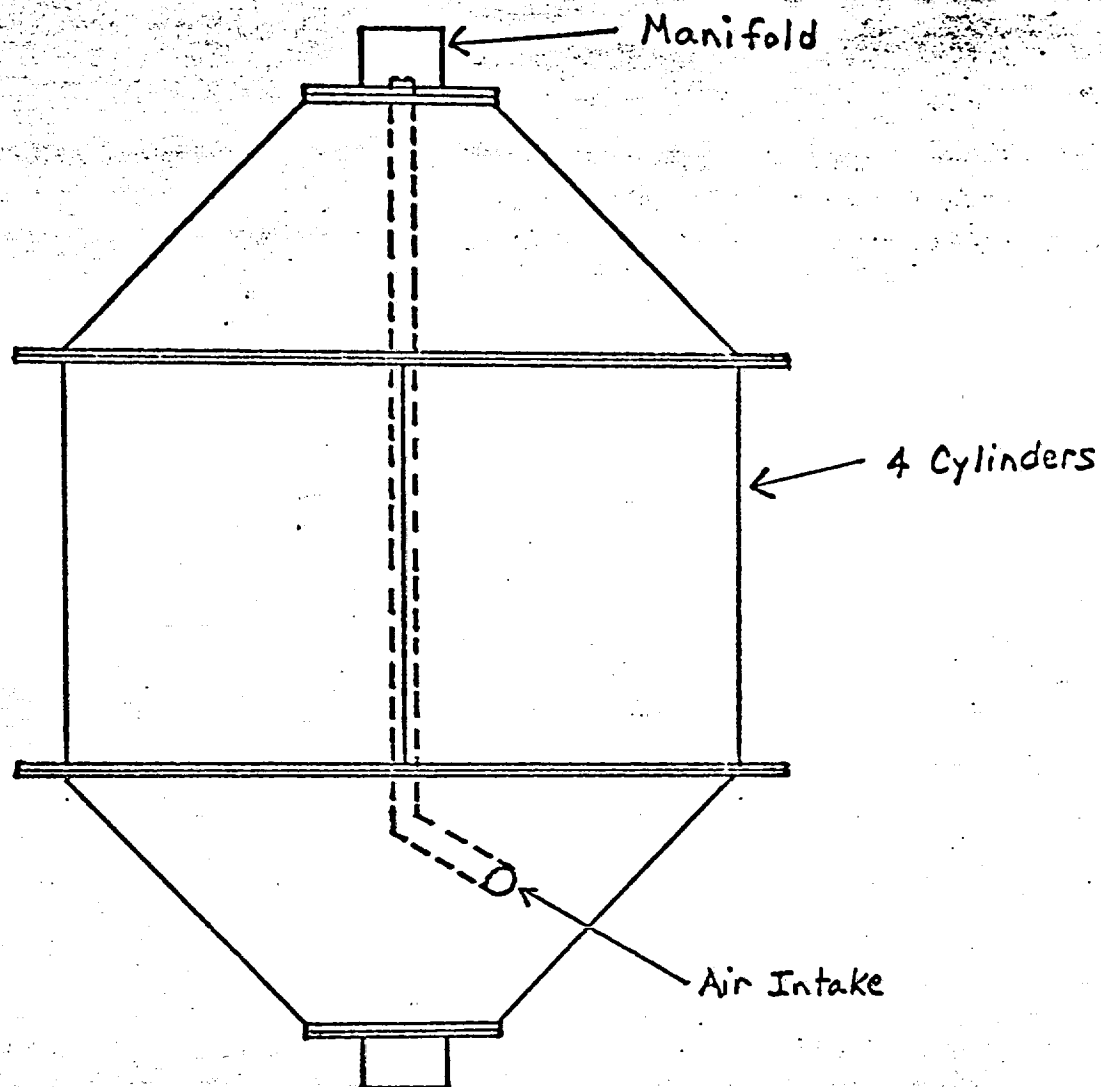
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Figure 3

Side View



Top View

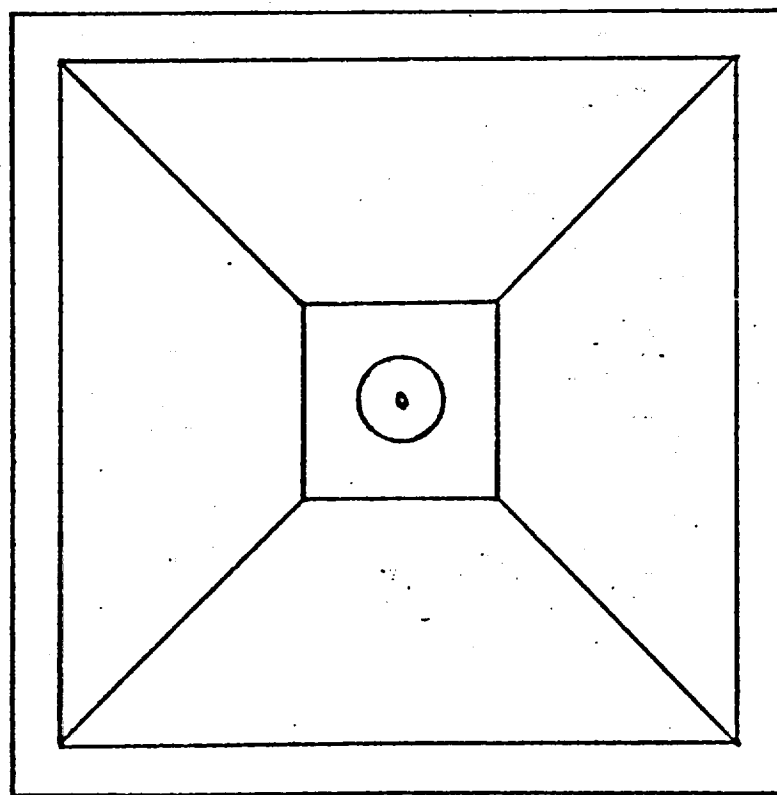


Figure 4

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1. Experiments on Biosynthesis and Turnover of Surfactant:

General: Adult male rats (Wistar or Long Evans) will be used and after initial treatment as outlined above each will be injected via the tail vein with isotopic mixtures as outlined below and killed by exsanguination by cardiac or aortic puncture at intervals from 1 to 150 hours following isotope injection. At sacrifice an endotracheal tube will be passed and surfactant obtained by lavage using 20 mM Tris (pH 7.5) in 1.15 M NaCl (35). Cells and debris will be removed from this wash by low speed centrifugation and surfactant obtained by high speed centrifugation as described by Pruitt et al (39). Cells will be examined microscopically and chemically in virus experiments. The lung, heart and liver will then be removed and carefully rinsed free of blood. For lipid analyses the tissues will be extracted as described previously (9). Lipids extracted from the various tissues will be analyzed using silicic acid column chromatography and thin layer chromatography to obtain pure triglycerides, 1,2 and 1,3-diglycerides lecithin and phosphatidyl ethanolamine (PE) by methods in standard use in this laboratory (8,9) and/or with two dimensional TLC (85). Purified lecithin and PE will be further sub-fractionated according to the degree of unsaturation of their fatty acids by argentation chromatography (8,86) or by mercuric acetate adduction (40). Completeness of these separations will be checked by gas liquid chromatography of the methyl esters as previously described (2). In studies of the methylation pathway monomethyl and dimethyl PE will be isolated (87,88). Radioactivity of the isolated compounds will be determined by liquid scintillation spectrometry using a toluene, a toluene-TritonX100 or a dioxane based scintillation fluid as appropriate (2).

2. Other Determinations to be made are the Following:

1. Tissue wet weights
2. Pool size of total lipids, total phospholipids, lecithins and phosphatidyl ethanolamines.

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3. Specific activities (dpm/ μ mole lipid phosphorus) of each phospholipid and their water soluble phosphorylated precursors.
4. Pool size of water soluble precursors if necessary by reverse isotope dilution as described (4).
5. Electron microscopic evidence of change in lamellar bodies, with autoradiography if indicated, appearance of surfactant lining layer and evidence of increased phagocytosis of lipid material by macrophages.

An aliquot of lung will be extracted as above, another aliquot taken for electron microscopy and the remainder homogenized and submitted to gradient centrifugation to obtain intracellular surfactant type lecithins as described by Pawlowski et al (36) or King and Clements (40). In other groups of rats similarly treated, suitable aliquots of lung, heart and liver will be taken for extraction in 70% ethanol for analysis of the water soluble precursors of lecithin and PE (phosphoryl choline, CDP-choline, phosphoryl ethanolamine and CDP-ethanolamine) as previously described (3,4). For electron microscopy tissue and surfactant will be processed as described by Finley et al (53).

Statistical analysis where applicable will be done using Students "t" test, the Mann-Whitney "U" test and isotope decay slopes will be drawn using the least square method with 95% confidence limits being ascertained for each slope. These programs have been computerized.

3. Specific Experiments: Turnover and Exchange Experiments:

Rats will be housed as described above and fed regular rat chow. The experimental group will be given synergistic mixtures of pollutants to breathe as outlined above. Control rats will be similarly housed and receive air instead of pollutants. After three or more days each rat will receive via tail vein suitable amounts of the isotopic compounds given below and then killed at intervals up to 150 hours. The results from these studies will be used to project longer time periods for future studies.

Based on our present data as reviewed in the Introduction, we believe that $2\text{-}^3\text{H}$ -glycerol and $1\text{-}^{14}\text{C}$ -palmitate (or $9\text{-}10\text{-}^3\text{H}$ -palmitate) are ideal tracers for surfactant turnover studies. These two isotopes will, therefore, be used as

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standard tracer substances. In addition, normal and pollutant exposed animals will also be studied using D_2 labeled palmitic acid. This stable isotope tracer has been chosen for the following reasons:

- 1) Because it is uniformly labeled, there are 32 deuterium atoms/molecule thus giving a very high signal to noise ratio on mass spectrometry.
- 2) Palmitic acid from surfactant and other lecithins can be readily separated by methylation and isolation of the methyl esters.
- 3) The methyl esters are volatile at temperatures used for mass spectrometry.
- 4) Methyl esters are readily quantitated by gas chromatography.

In addition to labeled glycerol and palmitate, experiments will be performed using ^{14}C -leucine to examine the relative relationship of lecithin and protein turnover in the surfactant system. The system will be isolated by either the method of Pruitt et al (39) or King and Clements (40). In view of the recent studies of Naimark (41) we will pay particular attention to the pulmonary alveolar macrophages. These cells will be isolated from lung lavage fluid (41) and their lipids extracted and analyzed as described above. Since such cells exhibit active phospholipid metabolism of their own, quantitative data on their catabolism of "ingested" surfactant may be difficult. The following approaches to this problem will be tried:

- 1) Comparison of total numbers of macrophages recovered in lung lavage fluid in normal and pollutant exposed rats at each time period (i.e. cells/mm³ of lavage fluid). A constant volume (3 x 6 ml) of fluid will be used for lavage.
- 2) Comparison of number of macrophages per alveolus as judged by light microscopy in normal and experimental animals. This method will be a check on #1.
- 3) DPL will be isolated from the macrophages in the lavage fluid and total dpm in DPL/lavage volume, as well as specific activity (dpm/ μ mole DPL) determined.

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These observations will be correlated with changes in metabolic rate of DPL in pollutant exposed animals in order to try to establish whether the changes in metabolic rates are secondary to stimulation of macrophage activity and/or macrophage population in the alveolae.

Studies will be performed to establish a standardized lavage procedure for recovery of surfactant lecithin. The procedure currently being used (39) yields fairly consistent recoveries of about 1 μ mole lecithin/rat, using 3 washes of 6 ml each. In order to be able to measure secretory rates more precisely it will be necessary to refine this method further. We will, therefore, compare the more recently published method of Young and Tierney (96) with our present system. By these means it should be possible to examine secretion rates of surfactant DPL in relation to pollutant exposures.

Since controversy still exists regarding the relative importance of the Kennedy and the methylation pathways to lecithin biosynthesis in the lung, it will be important to examine these two pathways after pollutant exposure, if exposure to pollutants results in more rapid turnover of surfactant DPL. Studies will be performed using methionine-methyl- ^3H , or ethanolamine-1-2- ^{14}C to evaluate the methylation pathway contribution, if any, to DPL biosynthesis in normal and experimental rats. Since this pathway is of established importance in liver, but of doubtful significance in lung, these studies will be performed in the isolated heart lung preparation to avoid any problems with uptake by lung of lecithin secreted by the liver into the circulation.

Based on the present rates of smoking of the rats it is not possible to predict what effect increased amounts of cigarette smoke or variable concentrations of other pollutants, etc. will have on the phospholipids in these tissues, especially since no relationship between pollutant concentrations and the amounts inhaled has been established. For this reason experiments in the larger chamber to be built will be more meaningful since a correlation between pollutant concentration in the chamber and amount inhaled should be more readily established. Experiments using various exposure times (current) must be coupled with varying concentrations.

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Some work with single pollutants will be carried out under the current NIH-grant. Of more interest will be studies with pollutants (such as SO_2) which are largely removed in the upper airways (89,90); if these produce a response (increased turnover rates after correction for increased respiratory rate, if any) an indirect effect must be operative; this could be sorted out by synergistic studies such as sulfur dioxide in the presence of oxidizing (transition metal salts) nuclei. Toxicity of long-term exposure to sulfuric acid mist has recently been reported (91).

Techniques for the preparation of controlled test atmospheres are routinely in use in one of our laboratories (ERA). These are done via dynamic syringe, rotating stopcock, permeation tube, and the use of plastic bags. Where possible continuous monitoring of pollutants will be performed (O_3 , NO, NO_2 -chemiluminescence; particles via CN-counter or mass sizing; SO_2 -via hydrogen peroxide or photometric).

Experiments with Essential Fatty Acid (EFA) deficient rats are currently underway. The combination of EFA-efficiency and pollutant exposure upon the synthesis, storage, and secretion of the surfactant system will be extended to other pollutant systems if present studies warrant it.

4. In Vitro Experiments

As phospholipid synthesis in cell free systems is not always the same as in intact animals, further studies will be performed using isolated heart-lung preparations. Such a system has been described in the rat by Simpson-Morgan (78). Heart-lung preparations prepared by this method will be obtained from normal and pollutant exposed animals and incorporation of suitable radioactive precursors after intracardiac injection followed for 1-4 hours in lung and heart by methods described above. These steps will permit study of surfactant synthesis in a system where hepatic synthesis of phospholipid and protein are excluded, thus allowing us

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to rule out any hepatic contribution. These studies will also avoid the problems associated with adipose tissue lipolysis secondary to nicotine stimulation.

Electron Microscopy will be used to evaluate the following:

1. To help ascertain purity of cell fractions.
2. To compare the appearance of macrophages in normal, EFA deficient and pollutant exposed animals looking particularly for evidence of phagocytosis of membrane structures resembling surfactant.
3. To examine the Type II alveolar cells in normal, EFA deficient and pollutant exposed animals with particular attention to the appearance both qualitatively and quantitatively of the lamellar bodies. It is hoped that such observation, coupled with biochemical data, will allow better understanding of the assembly and secretion of the surfactant system, particularly in relation to the membrane systems concerned with secretory granules and protein synthesis in EFA deficient animals.

D. SIGNIFICANCE

1. Do pollutants increase the turnover of surfactant lecithin? If this is the case the slope of disappearance from the surfactant of pollutant-exposed rats should be significantly greater than that of control animals. This is what we are finding currently with cigarette smoke. Long-term exposures of rats to high levels (2.9 ppm) of NO_2 resulted in a 13% decrease in lung compliance, a significant reduction in surface-active properties (increased surface tension) and an 8.7% decrease in lung lipid content, and a marked decrease in percentage of total saturated phospholipid fatty acids (51). It was suggested that lung instability (P-V measurements) with NO_2 -exposure was due to alterations in fatty acid composition of surfactant phospholipids. It is clearly desirable and necessary to combine turnover measurements with phospholipid fatty acid analysis from lavaged lungs if underlying mechanisms are to be understood. The experiments proposed should also shed light on the effect of pollutants on the phospholipid metabolism in heart and liver.

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2. Long term exposure studies are thought to be more significant than "forced" inhalation studies. Precise knowledge of the effects of various environmental factors upon the metabolism and function of the surfactant system is lacking. Epidemiological studies indicate that such factors play an important role in the pathogenesis of acute and chronic pulmonary disease in man.

Other factors have to be accounted for. Kerr (92) has shown that there is in man a diurnal variation of respiratory function independent of air quality; i.e. a chemical or metabolic change (for any observed changes) would be indicated in our experiments, rather than a mechanical one. However, we have not verified such a diurnal variation in the rat.

3. Which of the three pathways of lecithin synthesis is involved in surfactant production? In vivo and in vitro studies from control and pollutant exposed animals will determine which pathway shows increased activity in conformity with increased in vivo turnover. How do the findings relate to the surfactant pool?

4. Studies in man

Once the techniques for use of stable isotopic tracers have been developed based on the work done by Klein and co-workers (93,94) in the animal models described above we will apply their techniques to studies in healthy human subjects and patients with bronchopulmonary disease. Techniques for obtaining pulmonary lavage fluid for man have been developed by others and can be applied to these studies. Precise description of experiments in man will have to await more precise definition of the problem in animal studies. We anticipate that such human studies will not therefore be undertaken for at least two years. At that time a precise protocol will be submitted for review both by the granting agency and the Committees or Experimentation Involving Human Subjects of the Albany Medical College.

5. Clements (95) presented an interesting correlation of surface active material vs. pulmonary surface area for several species (experimentally and theoretically). Based on the theoretical curve all species have a surfactant reserve. This correlation (if the fashion in which it was arrived is accepted) will be important to the interpretation of turnover rates and pool sizes to be obtained from the proposed study.

E. FACILITIES AVAILABLE

The experimental laboratories available for this study include 1000 square feet of area for general medical research and 500 square feet of an air pollution laboratory. There are 350 feet of office area available.

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The following major items of equipment available for this study include:

liquid scintillation system (Beckman L S 250), preparatory ultracentrifuge (Beckman L 265), high speed refrigerated centrifuge (Servall RC 2-B), gas chromatograph (F & M Model 400), electron microscope (RCA-EMU), 2 fraction collectors, Packard strip counter, refrigerated microtome (Porter-Blum), gas chromatograph (Perkin-Elmer 900), gas chromatograph (Gow-Mac), ozone generator (Welsbach), ozone meter (Mast), spectrophotometers (B & L Spectronic 20, 70, 88), ultraviolet spectrophotometer (Beckman DU 2), NO_x analyzer (Scott 225 Chemiluminescence) atomic absorption spectrometer (Beckman), total carbon analyzer (MSA), integrating nephelometer (MR), condensation nuclei counter (Environment/One Rich 100), Lundgren impactor (Environmental Sciences), and radioactive Isotope measuring equipment. The following items are available -- to be shared with other investigators: Digital Equipment Corporation PDP-12 computer, NCR Century 200 computer, IBM 360/50 computer, nuclear magnetic resonance and electron spin resonance instrumentation, and mass spectrometry (Dr. Hudson's laboratory at RPI) and neutron activation analysis equipment.

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#976 - AYRES

1003539959

February 11, 1974

Grant application No. 976

PULMONARY

To: The committee comprising Drs. Gardner, Liebow, Wyatt

Subject: Stephen M. Ayres, M.D., St. Vincent Hospital, Worcester, Mass.
New application No. 976
"Relative Importance of Cigarette Smoking and Exposure to High Level Automotive Emissions to the Development of Chronic Bronchitis"

History

A preliminary inquiry was handled as Case No. 258.

Request

Application No. 976 requests \$30,061, plus two additional years.

Documents Submitted (copies enclosed)

1. Application dated 1/25/74 (8 pages)
2. C.V. and list of publications of Dr. Ayres
3. Face sheet, with abstract, of key publication; Arch. Env. Health 27: 168, 1973

(Full copy and other reprints will be sent if you wish.)

FWN:wg

FWM
F.W.N

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976

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 4 1974

Application for Research Grant

Date: 1/25/74

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Stephen M. Ayres, M.D.
Physician-in-Chief

2. Institution & address:

The St. Vincent Hospital
25 Winthrop Street
Worcester, Massachusetts 01610

3. Department(s) where research will be done or collaboration provided:

Medicine

4. Short title of study:

Relative Importance of Cigarette Smoking and Exposure to
High Level Automotive Emissions to the Development of
Chronic Bronchitis

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: Five Years

7. Brief description of specific research aims:

The specific aim of this study is to determine the relationships among cigarette smokers, exposure to automotive emissions and the development of chronic bronchitis. In addition, we plan to evaluate the frequency of physiologic abnormalities and symptoms in chronic bronchitis and to study the sequential development of chronic obstructive lung disease over an extended time period.

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Chronic bronchitis develops from the interaction of nature and nurture. A variety of inhaled materials, cigarette smoke and automobile exhaust, for example, may lead to small airway disease in genetically susceptible individuals. Small airway disease may or may not progress to symptomatic emphysema depending on the intensity of genetic and environmental factors.

9. Details of experimental design and procedures (append extra pages as necessary)

The appended reprint details a study of over 450 tunnel and bridge officers conducted over the past three years under contract from the Triborough Bridge and Tunnel Authority. This population group is composed of men who collect tolls and control traffic for seven bridges and two tunnels in New York City. These men are exposed to extremely high concentrations of automotive emissions and have relatively high levels of carboxyhemoglobin. We have shown that more than 75% of the group have laboratory evidence of small airway disease. A Venn diagram (see Figure 1) emphasizes the frequency of abnormal closing volumes and midexpiratory flow rates as well as the frequency of symptoms. While detailed smoking histories have not yet been evaluated, a similar incidence of bronchitis and decreased pulmonary function was observed in both smokers and non-smokers, leading us to question the belief that cigarette smoking is the major cause of small airway disease.

We are requesting support from the Council for Tobacco Research in order to specifically study the interrelationship between genetic abnormality and environmental exposure. The overall study will continue for another three years under contract with the Triborough Bridge and Tunnel Authority, but by virtue of an agreement with union and management, we are committed to study the incidence of coronary artery disease using treadmill and on-site electrocardiographic monitoring techniques.

Since considerable data on the prevalence of bronchitis has been already obtained, we are most anxious to continue the study of pulmonary disease in this group over a relatively long period of time.

We propose to further analyze the population by measuring the alpha I antitrypsin phenotype and by obtaining a detailed family history relating to the possible inheritance of pulmonary disease.⁽¹⁾ Aryl hydrocarbon hydroxylase inducibility will be measured by the technique of Kellerman et al,⁽²⁾ since these workers have recently shown an increase in the incidence of bronchogenic carcinoma in individuals with higher levels of inducibility suggesting a genetic predisposition.⁽³⁾ Complete

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9. Details of experimental design and procedures (cont'd)

hematologic evaluation, immunoelectrophoretic measurement of serum globulins and plasma complement, carboxyhemoglobin and blood lead measurements will be made as part of the overall study.

A population of over 400 men first studied in 1970 will be considered the experimental group. There is relatively little turnover in this group of workers and it is likely that the entire population can be followed for at least five additional years. We will make annual measurements of closing volume, mid-expiratory flow rate, airway resistance and functional residual capacity. These methods are detailed in the accompanying reprint. In addition, the British Research Council Respiratory Symptom Questionnaire and a postero-anterior and lateral chest x-ray will be repeated each year. This data will be stored in an in-house magnetic tape data system and be evaluated each year.

The following specific questions are part of the experimental design:

1. Are carcinoma of the lung, bronchitis or emphysema more common in tunnel workers than the general population?
2. Is there a dose effect between the development of respiratory disease and cigarette smoking in this population?
3. What is the relative importance of exposure to automotive pollution and cigarette smoking in the development of carcinoma of the lung, bronchitis and emphysema?
4. Is it possible to identify genetic markers which predict the development of respiratory disease in a heavily exposed population?

References

1. Lieberman, J., Mittman, C., and Schneider, A.S.: Screening for homozygous and heterozygous alpha₁-antitrypsin deficiency. *Journal of the American Medical Association*, 210, 1969.
2. Kellermann, Gottfried, Shaw, Charles R., Luyten-Kellerman, Mieke: Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *New England Journal of Medicine*, Vol. 289, No. 18, November 1973.
3. Kellermann, G., Luyten-Kellermann, M., Shaw, C.R.: Genetic variation of aryl hydrocarbon hydroxylase in human lymphocytes. *American Journal Human Genetics*, Vol. 25, 1973.

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5. 4
10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Computer facilities
Chemistry laboratory
St. Vincent Hospital
25 Winthrop Street
Worcester, Massachusetts

Pulmonary Function Laboratory
117 West 12th Street
New York, New York

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Please see attached list.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Please see attached list.

1003539964

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Ayres, Stephen M.

10%

0

Technical

Lab Technician

100%

\$9,000 + \$540

Computer Technician and Data
Analyzer

100%

\$10,000 + \$600

Sub-Total for A

$\$19,000 + \$1,140 =$
 $\$20,140$

B. Consumable supplies (by major categories)

Glassware, gases, chemicals

\$3,500

Questionnaire

\$ 500

Sub-Total for B

\$4,000

C. Other expenses (itemize)

Reprints and publishing

\$ 500

Computer

\$1,000

Travel

\$ 500

Sub-Total for C

\$2,000

Running Total of A + B + C

\$26,140

D. Permanent equipment (itemize)

Sub-Total for D

\$26,140

E

\$ 3,921

Total request

\$30,061

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | \$21,147 | \$4,200 | \$2,000 | 0 | \$4,102 | \$31,444 |
| Year 3 | \$22,207 | \$4,400 | \$2,200 | 0 | \$4,321 | \$33,128 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| | | | |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---------------------|---|----------------------|--------------------|
| Health Surveillance | Triborough Bridge and Tunnel Authority | \$70,000 per year | 1/24/72-1/24/76 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Stephen M. Ayres, M.D.

Signature Stephen M. Ayres Date 1-30-74

Telephone 617 798-6177
Area Code Number Extension

Checks payable to

St. Vincent Hospital

Mailing address for checks

25 Winthrop Street
Worcester, Massachusetts 01610

Responsible officer of institution

Typed Name Miss Helen Marie Smith

Title Executive Director

Signature Helen Marie Smith Date 1-30-74

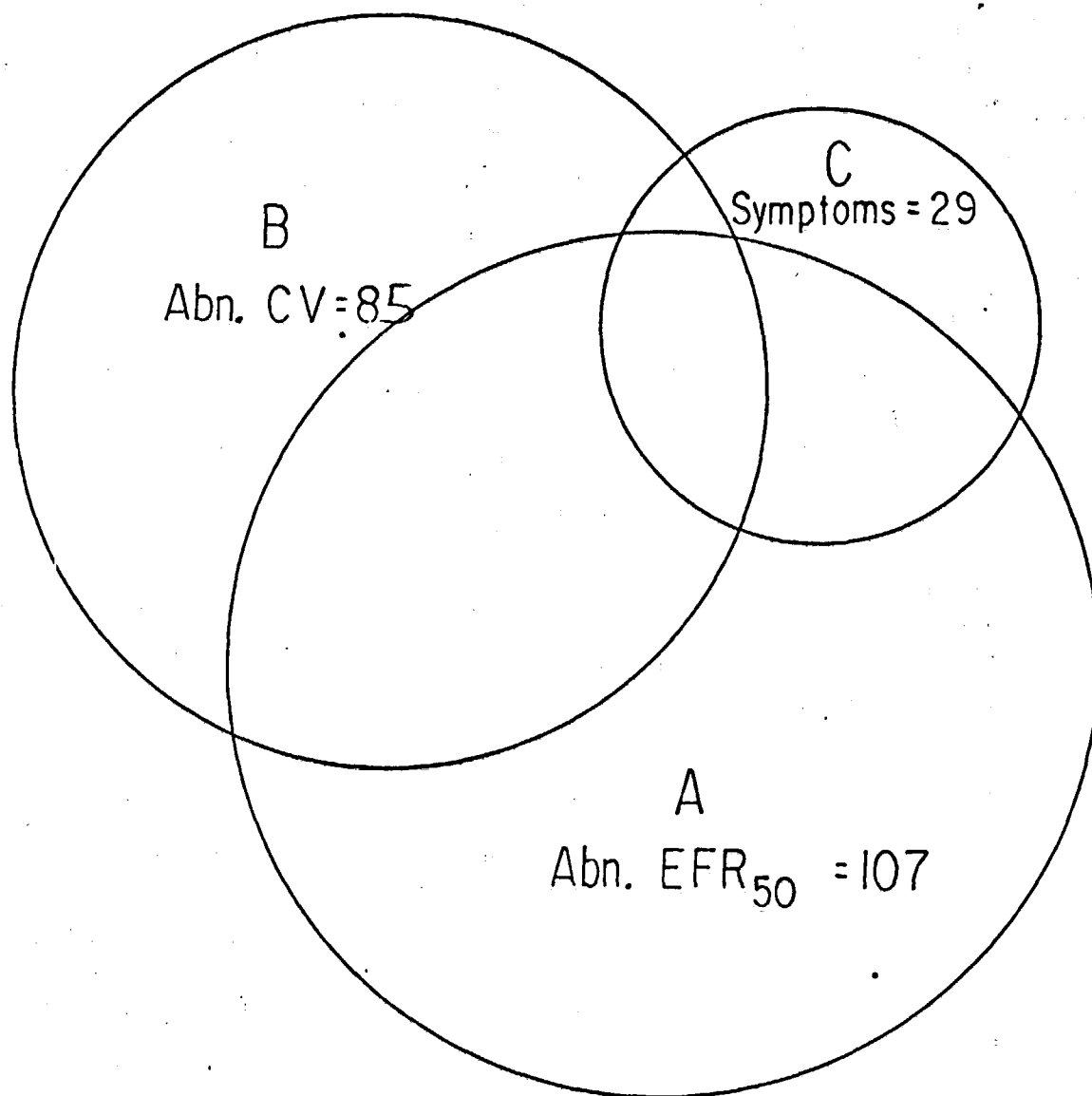
Telephone 617 798-6066
Area Code Number Extension

1003539966

13. Publications:

1. Ayres, Stephen M., Mueller, Hiltrud S., Gregory, John J., Giannelli, Stanley, Jr., and Penny, John L.: Systemic and myocardial hemodynamic responses to relatively small concentrations of carboxyhemoglobin (COHB). Archives of Environmental Health, Vol. 18, April 1969.
2. Ayres, Stephen M., and Buehler, Meta E.: The effects of urban air pollution of health. Clinical Pharmacology and Therapeutics, Vol. 11, No. 3, May-June 1970.
3. Ayres, Stephen M., Giannelli, Stanley, Jr. and Mueller, Hiltrud: Myocardial and systemic responses to carboxyhemoglobin. Annals of The New York Academy of Sciences, Vol. 174, Article 1, October 1970.
4. Ayres, Stephen M., Giannelli, Stanley, Jr., and Mueller, Hiltrud: Carboxyhemoglobin and the access to Oxygen. An example of human counterevolution. Archives of Environmental Health, Vol. 26, January 1973.
5. Ayres, Stephen M., Evans, Robert, Licht, David, Griesbach, Jane, Reimold, Felicity, Ferrand, Edward F. and Criscitiello, Antoinette: Health effects of exposure to high concentrations of automotive emissions. Studies in bridge and tunnel workers in New York City. Archives of Environmental Health, Vol. 27, September 1973.

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Figure 1 - Distribution of abnormally low mid-expiratory flow rates (EFR₅₀), increased closing volumes (CV) and symptoms of chronic bronchitis in a population of 111 non-smoking bridge and tunnel workers.

Health Effects of Exposure to High Concentrations of Automotive Emissions

Studies in Bridge and Tunnel Workers in New York City

Stephen M. Ayres, MD; Robert Evans; David Licht; Jane Griesbach;
Felicity Reimold; Edward F. Ferrand, PhD; Antoinette Criscitiello, RN, MA, New York

On-site and remote health evaluations were performed on 550 employees of the Triborough Bridge and Tunnel Authority in New York City. Extremely high ambient air pollution was observed. Carbon monoxide averaged 63 ppm over a 30-day period with a maximum hourly concentration of 217 ppm in one facility. Eighty-five percent of the smoking and 47% of the nonsmoking tunnel workers had carboxyhemoglobin saturations in excess of 3%. A high percentage of the group had symptoms suggestive of chronic bronchitis; airway resistance was elevated in one third and almost all bridge and tunnel workers had an increase in closing volume, suggesting small airway disease.

Automotive pollution produced by uncontrolled emissions from multiple mobile sources has rapidly become the major air pollution problem in most urban areas. Carbon monoxide, nitrogen oxides, hydrocarbons, and oxidants are generated in large volume by the internal combustion engine and are all potentially hazardous to human health. The lack

of systematic epidemiological observations and the ethical difficulty associated with large scale experimental exposure have prevented identification of precise dose-response relationships. A group of recent studies has suggested that carboxyhemoglobin concentrations in excess of 5% may induce myocardial ischemia in subjects with coronary artery disease¹⁻³ and long-term breathing of nitrogen dioxide concentrations averaging 2.9 ppm has been shown to produce significant pulmonary abnormalities in laboratory animals.⁴ The aggregate effect of automotive pollution on human health was clearly demonstrated by Aronow et al⁵ who exposed ten patients with angina pectoris to 90 minutes of Los Angeles freeway traffic. Carboxyhemoglobin saturation rose from 1.12% to 5.08%, four of the subjects developed electrocardiographic abnormalities, and the average time to develop angina with treadmill exercise fell from 249.4 to 174.3 seconds. The amount of exercise required to produce angina was significantly lower than control two hours later, when carboxyhemoglobin concentration had fallen to 2.91% saturation.

Bridge and tunnel workers, policemen, taxi drivers, and others comprise an unwitting but useful test population. Exposed daily to high

concentrations of automotive pollutants, they might be considered an exaggerated model of the environmental stresses experienced by many other city dwellers. Differences between industrial and community air pollution exposures blend as the commuter travels each day through congested tunnels and highways. While an eight-hour tour of duty is considered an occupational exposure to the tunnel workers, a two-hour daily traffic period for the New Yorkers is generally considered a problem of community air pollution. Obviously, rigid distinctions between occupational and community exposures are arbitrary, and it is likely that environmental and occupational air quality standards will ultimately be similar.

The interest of the Bridge and Tunnel Officers Union and the Triborough Bridge and Tunnel Authority in the problem of automotive pollution has provided a unique opportunity to study the effects of automotive pollutants on human health. A three-year study of all nonadministrative employees of the Authority was initiated in 1970; this article details information collected during the first two years of observation. An extensive program of toll booth air purification was completed during the early months of the second year of study.

Submitted for publication June 29, 1973; accepted July 2.

From the departments of medicine, St. Vincent's Hospital and New York University School of Medicine, New York.

Read before the Air Pollution Medical Research Conference of the American Medical Association, Chicago, Oct 3, 1972.

Reprint requests to Department of Medicine, St. Vincent's Hospital and Medical Center, 25 Winthrop St, Worcester, MA 01610 (Dr. Ayres).

7/6/80 - COCHISE

1003539970

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1974

Grant application No. 764C

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Gardner, Liebow and Wyatt

Subject: Charles G. Cochrane, M.D., Scripps Clinic and Research Foundation,
LaJolla, California

Continuation application No. 764C

"The Mediation of Inflammatory Injury of Tissue"

History

CTR has supported this study since 1970.

Last year Dr. Cochrane submitted a three-year request at approximately \$38,000 per year. A terminal grant for one year at \$11,000 was offered and accepted on that basis.

Hence the enclosed proposal competes without commitment.

Request

Application No. 764C requests \$16,560, for one year only.

Document Submitted

Enclosed is application dated February 1, 1974, incorporating summary progress report.

FWN:gh

Encls.

F.W.N.
F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 7 1974

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒

Second Renewal ☐

Date: February 1, 1974

1. Principal Investigator (give title and degrees):

Charles G. Cochrane, M. D. - Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Experimental Pathology

4. Short title of study:

The Mediation of Inflammatory Injury of Tissue

5. Proposed renewal date: July 1, 1974

6. How results to date have changed earlier specific research aims:

The enzymatic constituents of alveolar macrophages have been found to activate Hageman factor and with it, the intrinsic clotting, kinin forming and fibrinolytic systems. Our attention will now be directed toward analysis of the enzymes involved and their mechanism of release from the alveolar macrophages. In addition, Dr. John Craighead (personal communication) has observed kaolin in alveolar macrophages of smokers. Since kaolin is an excellent activator of Hageman factor, we will determine the relationship of aerosolyzed, inhaled kaolin, alveolar macrophages and pulmonary inflammation.

7. How results to date have changed earlier working hypothesis:

Earlier hypotheses have not changed.

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8. Any additional facilities now required? Describe briefly:

No new facilities are required.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

1003539973

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. Cochrane, C. G., Revak, S. D. and Wuepper, K. D., Activation of Hageman factor in solid and fluid phases. A critical role of kallikrein. *J. Exp. Med.*, 138: 1564, 1973.
2. Wuepper, K. D., Prekallikrein deficiency in man. *J. Exp. Med.*, 138: 1331, 1973.
3. Henson, P. M., Mechanism of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin: An EM study. *Arthritis and Rheum.*, 16: 208, 1973.
4. Cochrane, C. G., Revak, S. D., Aikin, B. S. and Wuepper, K. D., The structural characteristics and activation of Hageman factor, In *Inflammation, Mechanisms and Control*, (Lepow and Ward, eds), Academic Press, 1972, p. 119.
5. Wuepper, K. D., Biochemistry and biology of components of the plasma kinin forming system, In *Inflammation, Mechanisms & Control*, (Lepow and Ward, eds), Acad. Press, 1972, p. 93.

(continued)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

2a

11. Publications (continued)

6. Johnston, A., Cochrane, C. G. and Revak, S. D., The relationship of Pf/dil and activated human Hageman factor., J. Immunol., 112: 1974.
7. Ulevitch, R., Letchford, D. and Cochrane, C. G., A direct enzymatic assay of activated Hageman factor, Thrombosis et Diath. Hemorr., March, 1974.
8. Russell, S. and Cochrane, C. G., Cellular events associated with regression and progression of murine (Moloney) sarcoma in mice. Int'l J. Cancer, March, 1974.

1003539974

10. Appended outline of experimental protocol for ensuing year.

- A. Experimental activation of the intrinsic clotting, kinin forming and fibrinolytic systems in lung. Alveolar macrophages will be isolated from lungs of rabbits by lavage (Brain et al. Arch. of Int. Med. (26:477, 1970), a technique already employed in this laboratory, and the granules obtained by disruption of the cells in hypertonic sucrose. After lysis of the granules, the constituents will be assayed for their capacity to activate Hageman factor over a range of conditions of pH, temperature and ionic strength. Since Hageman factor, activated by enzymes such as plasmin or kallikrein is cleaved in the process, a similar effect of the granular constituents of alveolar macrophages will be examined. This will define the mechanism of activation as fluid phase rather than solid phase (i.e., when Hageman factor binds to negatively charged surfaces such as that provided by alveolar membranes). The implication would be that clotting of plasma, and release of kinins and plasmin would occur in the fluid of the alveolar spaces, thereby provoking a diffuse reaction.

The granular constituents of the alveolar macrophages will then be fractionated on DEAE Sephadex A50 under conditions of constant pH and varying salt concentration. Enzymes or other activators of Hageman factor will be measured and isolated and identified through additional chromatographic and physical methods.

To determine the rate and extent of activation of Hageman factor and its related components (prekallikrein, clotting factor XI and plasminogen), these proteins will be radiolabelled and injected intravenously in rabbits. The disappearance from the circulation and accumulation in alveolar tissues and lavage-washings will be measured quantitatively. By SDS acrylamide gel electrophoresis under reducing conditions, activation of the Hageman factor, prekallikrein, plasminogen and factor XI will be determined since the molecules are cleaved during fluid phase activation (J. Exp. Med., 138:1564, 1973). These measurements will be made in rabbits receiving various forms of pulmonary injury: 1) rabbits will be injected with goat antibody directed specifically to basement membrane of lung to induce injury; 2) rabbits will be exposed to aerosolyzed finely particulate kaolin. Dr. Paul Gross has expressed willingness to help in these experiments; 3) immunologic complexes of bovine-albumin(BSA)-anti BSA will be prepared in an excess of antigen (J. Exp. Med., 118:489, 1963) and injected intravenously. These complexes deposit in the pulmonary vasculature and induce acute inflammation involving complement, neutrophils and platelets; 4) IgE mediated active systemic anaphylaxis will be produced in rabbits which causes release of vasoactive amines both intravenously and from the mast cells of the pulmonary beds.

In each of these experimental conditions the following examinations will be performed:

1. Careful histologic examination of the inflammatory reaction in the lung.
2. Fluorescent antibody localization of Hageman factor and kallikrein in the tissues. The presence or absence of fibrin will also be determined.
3. Kinetic analysis of the disappearance of radiolabelled components from the circulation as noted above.

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1c

10. Appended outline of experimental protocol for ensuing year (page 2)

4. Accumulation of radiolabelled components in the pulmonary bed as compared with accumulation in other organs.
5. Analysis of the component molecules with SDS acrylamide gel electrophoresis to detect cleavage and thereby evidence of fluid phase activation.

These experiments are directed at the mechanism of activation of Hageman factor and its related systems in pulmonary inflammatory disease. Solid phase (localized) activation of Hageman factor will be detected by fluorescent antibody techniques and fluid phase (diffuse) activation by analysis of cleaved Hageman factor in the lavage fluid. It must be emphasized that these events may be of great importance in the injury of lung, the deposition of fibrin and stimulation of collagen formation. Perhaps the best studied parallel to this series of events is found in glomerulonephritis where sclerosis of the glomerulus may be directly related to the deposits of fibrin in Bowman's space.

B. Activation of Hageman factor and its related systems by human alveolar macrophages.

1. Using alveolar macrophages obtained from the pulmonary division of the Department of Medicine, University of California, San Diego, constituents capable of activating members of the Hageman factor systems will be sought along lines similar to those noted above in the rabbit studies. The cells will be provided by Drs. J. Clausen and Anthony Catanzarro under collaborative studies already established.
2. Following the observation by Craighead and his associates, we will determine if kaolin particles are present in the macrophages by electron microscopy and if the presence of kaolin is associated with smoking. According to Craighead, a potential source of the kaolin is the tobacco leaves which are apparently dried with kaolin as a drying agent. The macrophages will be lysed in NaOH, and the particles isolated and tested for their ability to bind and activate ^{125}I human Hageman factor. This is a property of kaolin that can be measured with exquisite sensitivity. Hageman factor already bound to the particles obtained from the macrophages will be sought in addition, although the likelihood of this is poor since the phagocytized particles would almost certainly lose bound proteins.

In addition, normal alveolar macrophages will be exposed to kaolin in the presence of ^{125}I Hageman factor. Release of activated, cleaved Hageman factor will be measured using acrylamide gel electrophoresis in order to determine the extent of fluid phase activation of Hageman factor occurring during the phagocytic process. This will shed light on potential diffuse release of injurious mediators.

1003539976

12. Summary progress report.

1. Structural studies of Hageman factor, the first component of the intrinsic clotting, kinin forming and fibrinolytic systems.

Hageman factor was found to be activated by two mechanisms. The first, occurring in solid phase is induced by molecular conformational changes associated with the binding of the molecule to negatively charged surfaces. Such insoluble materials as kaolin or vascular basement membranes and collagen serve to bind and activate Hageman factor. Preliminary evidence indicates that cut sections of pulmonary tissue also bind Hageman factor. In the second method of activating Hageman factor, enzymes derived from plasma and tissue serve to activate the molecule in fluid phase. Kallikrein, plasmin, factor XI and enzymes derived from alveolar macrophages were all found active in this regard. In fluid phase activation, Hageman factor is cleaved into fragments of 52,000, 40,000 and 28,000 MW. The 28,000 MW fragment bears enzymatic activity, and in turn is responsible for the activation of prekallikrein and factor XI of the intrinsic clotting system.

In order to obtain significant activation of the three systems, prekallikrein was found to be essential. In its absence, Hageman factor in plasma would not trigger the intrinsic clotting and fibrinolytic system to a degree adequate for measurement. These studies were made possible by the identification of a plasma obtained from individuals genetically deficient in prekallikrein - the so-called Fletcher trait plasma. Studies in this laboratory identified the missing factor as prekallikrein (1, 2).

2. Mechanisms of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin.

The reaction of human neutrophils with immunologic complexes or aggregates was found to depend upon specific surface receptors. If the aggregates were of a suitable size, phagocytosis ensued: particles were engulfed and granules released into the phagocytic vacuole. A proportion of potentially injurious granule constituents, however left the neutrophil through mechanisms which do not involve cell lysis but appear to be a consequence of the normal degradation phenomenon. Thus, if for any reason the vacuole was, or later became, open to the outside, there was extracellular release of the granules' enzymes. By means of electronmicroscopy, four possible mechanisms were demonstrated which might morphologically account for the escape of vacuolar contents from human neutrophils phagocytosing aggregated γ -globulin. According to the data, there was no release of lactic dehydrogenase and the cells were not lysed. Extrusion of granules seemed to occur primarily into vacuoles. Such vacuoles, however, occasionally had direct access to the exterior, as for example: (1) when they apparently opened to allow additional material to be ingested; (2) when they remained connected to the exterior by a narrow slit; (3) when two cells were involved in phagocytosis of a single particle (aggregate); (4) when extrusion of granules preceded the complete closing of pseudopods around the aggregates.

1003539977

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Charles G. Cochran

20

-0-

Peter M. Henson

20

-0-

Stephen W. Russell

50

9,000

Technical

Part time services of animal caretaker and dishwasher

2,500

Sub-Total for A

\$11,500

B. Consumable supplies (by major categories)

Chemicals, proteins

1,000

Glassware, plasticware

900

Animals, feed and bedding

1,000

Sub-Total for B

2,900

C. Other expenses (itemize)

none

Sub-Total for C

Running Total of A + B + C

\$14,400

D. Permanent equipment (itemize)

none

Sub-Total for D

E

\$2,160

E. Indirect costs (15% of A+B+C)

Total request

\$16,560

1003539978

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|-------------------------------------|-----------|--------------------|
| Immunologic Studies | United States Public Health Service | \$150,000 | 1974-1975 |
| The Mediation of Inflammatory Injury of Tissues | Council for Tobacco Research | \$16,500 | 1973-1974 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
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It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

O. K. Kincaid, Jr.

Mailing address for check:

Scripps Clinic and Research Foundation

476 Prospect Street, La Jolla, CA 92037

Principal investigator

Typed Name Charles G. Cochrane, M. D.

Signature Charles G. Cochrane Date Feb. 2, 1974

Telephone (714) 459-2390 x-306

Area Code Number Extension

Responsible officer of institution

Typed Name Frank J. Dixon, M. D.

Title Chairman, Biomedical Research Departments

Signature Frank J. Dixon Date Feb. 4, 1974

Telephone (714) 459-2390 365

Area Code Number Extension

1003539979

#954 - COHEN

1003539980

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

January 25, 1974

Grant application No. 954

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Gardner, Liebow and Sommers

Subject: Allen Barry Cohen, M.D., Ph.D., University of California Service,
San Francisco

New application No. 954

"The Genetic Defect in Alpha-1-Antitrysin Deficient Patients"

History

Dr. Cohen's current CTR grant "Human Alveolar Macrophages and Emphysema" ends March 31, 1974. The enclosed application is submitted as a "new" proposal and is indeed a different project.

Application #954 requests \$46,045 for the first year. Two succeeding years are estimated at \$59,152 and \$63,215. This increase is defended in "BUDGET COMMENTARY", page 6 of the application.

Dr. Cohen requests an April 1, 1974 starting date, to coincide with termination of his current CTR grant.

Document Submitted

Attached is application dated January 10, 1974: 22 pages plus CV and publications of Drs. Cohen and Lo.

Comment

Enclosed is a copy of Dr. Gardner's memo dated July 25, 1973 on his July 12 site visit to this grantee.

FWN:gh

Encl.

FWN
F. W. N.

1003539981

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

JAN 25 1974

Application for Research Grant
(Use extra pages as needed)

Date:

January 10, 1974

1. Principal Investigator (give title and degrees):

Allen Barry Cohen, M.D., Ph.D.
Assistant Professor of Medicine in Residence

2. Institution & address:

University of California Service
Chest Division
San Francisco General Hospital
San Francisco, California 94110

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study:

The Genetic Defect in Alpha-1-Antitrypsin Deficient Patients

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

The occurrence of alpha-1-antitrypsin deficiency in some patients with familial emphysema is one of the few clues available to the nature of the biochemical processes in human emphysema. The purpose of these experiments is to determine the biochemical basis for the genetically determined differences between the alpha-1-antitrypsin of normal subjects and that of subjects with the genetic variant of alpha-1-antitrypsin which predisposes some of them to emphysema. These studies may enable us to determine whether the abnormality in alpha-1-antitrypsin is the primary defect in these individuals or whether there are other defects as well. In addition, if it can be concluded that other defects are likely to exist, this investigation may suggest experiments to discover those defects.

1003539982

8. Brief statement of working hypothesis:

2.

The purpose of these experiments is to differentiate between two alternative hypotheses:

A) The abnormal gene which leads to a deficiency of alpha-1-antitrypsin in the blood is the structural gene for alpha-1-antitrypsin which codes for the alpha-1-antitrypsin polypeptide.

B) The abnormal gene controls some aspect of alpha-1-antitrypsin other than the primary sequence of amino acids such as the number and type of charged carbohydrate moieties on the molecule.

9. Details of experimental design and procedures (append extra pages as necessary)

Please see attached pages, beginning at Page 7.

1003539983

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities:

Laboratories at the San Francisco General Hospital, Building 100, Room 272 and 273. (Room 272 is on loan for an indefinite time.)

Space:

Room 272 = 185 sq.ft.

Room 273 = 480 sq. ft.

Equipment:

Centrifuges: Sorval RC3, Beckman Model L3-50 preparative ultracentrifuge

Electrophoretic equipment: Immuno-electrophoresis, starch block, pevikon block, preparative polyacrylamide gel, analytic polyacrylamide gel, thin layer, and two current or voltage regulated power supplies.

Fraction Collectors: Two ISCO fraction collectors with UV monitors.

Beckman Kintrac single beam spectrophotometer.

Radiometer pH Stat and Beckman pH meter
(list continues on page 3a)

11. Additional facilities required:

No additional space required.

(see Equipment in budget request)

12. Biographical sketches of investigator(s) and other professional personnel (append):

See appended curriculum vitae for A. Cohen and T. Lo.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached reprints.

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Lyophilizer

Assorted refrigeration equipment and incubators

Tissue culture hood

Distillation and rotary evaporation equipment

Zeiss phase fluorescent photomicroscope

Isoelectric focusing apparatus

Hewlett-Packard model 9100 calculator

Photovolt densitometer

Mettler HT-20 micro balance and Mettler P1200 macro balance

Water baths

Other available facilities:

Packard gamma and beta isotope spectrometer (jointly owned
with Dr. John Murray)

Gas-Liquid chromatograph (available from Dr. John Clements)

Cold Room Facilities

(shared facilities with
other University researchers
located at S.F.G.H.)

Beckman HP119 amino acid analyzer

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Allen B. Cohen, M.D., Ph.D.

30%

-0-

Theresa Lo, Ph.D.

100%

-0-

Technical

"to be recruited"

Staff Research Associate II, Step 2

100%

\$13,317

Sub-Total for A \$13,317

B. Consumable supplies (by major categories)

| | | | |
|--|--------|-------------------------|--------|
| Enzymes | \$2000 | Ampholytes (25 runs) | \$ 500 |
| Substrates | \$1500 | General Chemicals | \$1500 |
| Column resins | \$ 800 | Pipettes (vol. & micro) | \$ 500 |
| Glassware | \$1000 | Isotopes | \$ 700 |
| Miscellaneous laboratory and office supplies | | | \$1200 |

Sub-Total for B \$ 9,700

C. Other expenses (itemize)

| | |
|---|--------|
| Animals for immunization | \$2000 |
| Blood for alpha-1-antitrypsin preps (200 units) | \$5000 |
| Publication costs (reprints & page charges) | \$1500 |
| Service contracts on equipment | \$2000 |
| Travel (\$500 for each professional) | \$1000 |

Sub-Total for C \$11,500Running Total of A + B + C \$34,517

D. Permanent equipment (itemize)

| | |
|--|--------|
| ISCO Fraction collector and monitoring system | \$3300 |
| Fluid pumps (2) | \$ 800 |
| Haake Model FK water bath with heating, cooling, and external circulation | \$ 750 |
| American Instruments Co. fluorocolorimeter | \$1500 |

Sub-Total for D \$6,350

E. Indirect costs (15% of A+B+C)

E \$5,178Total request \$46,045

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | 27,707 | 10,475 | 11,950 | 1500 (misc.) | 7520 | \$59,152 |
| Year 3 | 29,862 | 11,313 | 12,425 | 1575 | 8040 | \$63,215 |

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Yr. | Inclusive Dates |
|---|---|--------|-----|--------------------|
| The role of the human alveolar macrophage in the production of emphy- sema | Council for Tobacco Research-USA, Inc. | 16,348 | 1 | April, 1971 |
| | | 16,407 | 2 | to |
| | | 12,427 | 3 | March, 1974 |
| | | 45,742 | | |
| Alpha-1-antitrypsin: Does it inhibit an enzyme which causes emphysema? | USPHS, NHLI HL 14201 | 32,635 | 74 | June, 1971 |
| | | 34,265 | 75 | to June, 1976 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--------------------------------|--------|-----------------------------------|
| The genetically determined biochemical defect in patients with emphysema and alpha-1-antitrypsin | USPHS, NHLI HL 06285 | 80,000 | 75 July, 1975 to July, 1982 |
| (See Budget Commentary, item #1 regarding this grant.) | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accepted the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Allen B. Cohen, M.D., Ph.D.Signature Allen B. Cohen Date 1-10-74Telephone 415 648-8200 ext. 273 or 527
Area Code Number Extension

Checks payable to

Regents of the University of Calif.

Mailing address for checks

1487 Fourth AvenueSan Francisco, Calif. 94143

Responsible officer of institution

Typed Name Ms. Sue ClarkTitle Program Coordinator
Gifts & Endowments OfficeSignature Sue Clark Date 1/22/74Telephone 415 666-2047
Area Code Number Extension

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BUDGET COMMENTARY

1. As shown on page 5 of the application, a grant submitted to the National Institutes of Health is pending. The grant is similar to this application but would start one year later if it is awarded. If both grants are funded to the extent requested, the renewal of the Tobacco Research Council grant will not be requested for the second and third years.

2. On his site visit last Fall, 1973, Dr. Gardner said that those investigators working on an April 1st grant year could be awarded grants beginning April 1st. If this is possible, I would appreciate having the April 1st starting date, with appropriate adjustment to the 12 month budget submitted.

3. Dr. Theresa Lo's salary will be paid by a Cardiovascular Research Institute Postdoctoral Traineeship until July, 1975. After this date Dr. Lo will continue to work as part of our permanent staff. During the entire time the experiments described in this protocol will occupy her full time. However, it will be necessary to pay her salary from this grant subsequent to July, 1975, and this is included in the Year 2 salary budget (salary, \$11,325 plus staff benefits, \$1,699 = total \$13,024).

4. Fraction Collector and Monitoring System: This laboratory utilizes two fraction collectors nearly full time. In order to perform the numerous peptide and heterosaccharide separations required by the protocol, another fraction collector and monitor will be necessary. The equipment requested in this application interfaces with our existing fraction collectors and monitors from ISCO. They were selected because the dual beam optical unit has the sensitivity which will be required for the small amounts of ZZ protein which will be available and because the optical unit also reads absorbance at wave lengths necessary for monitoring ninhydrin reactions.

5. Fluoro-colorimeter: This instrument is necessary for measuring the extremely small amounts of protein from thin layer chromatograms, polyacrylamide gels, etc. With this instrument, using the method of Bohlen and his colleagues (Arch. of Biochem. 155:213, 1973), proteins, polypeptides, or amino acids can be measured in the nanogram range. In addition, quantitative analysis of dansylated amino acids is possible.

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9. Details of experimental design and procedures.

THE GENETIC DEFECT IN ALPHA-1-ANTITRYPSIN DEFICIENT PATIENTS

Allen B. Cohen, M.D., Ph.D.

A). Background and rationale: AAT deficiency is a genetically controlled disease characterized by a predisposition to pulmonary emphysema and low levels of AAT in the blood. AAT is an alpha-1-globulin in serum which inhibits trypsin, chymotrypsin, porcine pancreatic elastase, plasmin (1), thrombin (2), proteases from human neutrophils (3) and human alveolar macrophages (4). The protein has a molecular weight of 52000 (1) contains 2, half-cystines per mole., and probably has one polypeptide chain (5). In 1965 Gross and his colleagues demonstrated that an emphysema-like pathologic appearance developed a short time after the injection of papain into the tracheas of guinea pigs (6). Erikson (7) suggested the hypothesis that proteolytic enzymes from granulocytes and macrophages may destroy the parenchyma of the lung in AAT deficient patients because these enzymes are not adequately inactivated. Most research on the etiology of emphysema in AAT deficient patients has focused on this hypothesis. However, many important alternative possibilities have been bypassed even though there was no scientific reason to exclude them. The purpose of this protocol is to explore some of these possibilities by attempting to determine which protein is coded for by the abnormal gene in patients with this disease.

Studies of the genetics of AAT deficiency were made possible by the experiments of Fagerhol who developed a method of acid starch gel-immunocrossed gel electrophoresis for demonstrating that AAT differed qualitatively as well as quantitatively in patients with this disease (8). This qualitative difference is demonstrated by electrophoresing serum in low pH buffers in hydrolyzed starch blocks. The strip of starch containing the alpha-1-globulins is removed and placed on a thin slab of agar on a glass plate. The agar is made up with alkaline buffers and contains antibody against AAT. The alpha-1-globulins are electrophoresed across the agar gel and the alpha-1-antitrypsin precipitated in a pattern of peaks which characterizes the phenotype of the individual. The results of these studies were compatible with the hypothesis that the phenotype of AAT is controlled by a series of codominant alleles and the genes from both parents are expressed in the phenotypic pattern (8). The phenotypic pattern designated pi type (proteinase inhibitor type) MM, characterized by 6 peaks, is

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manifested by the alpha-1-antitrypsin from most people and the phenotype pi AA, characterized by 4 peaks, is manifested by AAT from individuals who developed emphysema at an early age (6,9,10). The relationship of other phenotypes to lung disease is not proven, and therefore, they will not be studied in these experiments.

While the differences between the electrophoretic patterns of AAT from individuals with Pi types MM and ZZ have been clearly shown, the functions of the gene which causes the differences between these two electrophoretic patterns have not yet been determined. (In the ensuing discussion two types of heterogeneity of AAT will be examined: heterogeneity between individuals of different phenotypes, and heterogeneity within a single person. The first type of heterogeneity is the subject of this protocol, and the second type of heterogeneity will be examined only to the extent necessary to determine the structural basis of the differences between phenotypes.) An understanding of the structural differences between AAT of different phenotypes may suggest the function of the abnormal protein. For example, if MM and ZZ protein differed only by a specific carbohydrate moiety, then the defective gene may code for a sugar transferase which adds sugar moieties to the completed polypeptide. This possibility was suggested by the experiments of Bell and Carrell (11). These investigators showed that during digestion of MM protein with neuraminidase, the phenotype of MM protein passes through a phase in which it resembles the ZZ protein on acid starch gel-immuno-crossed gel electrophoresis. They suggest that a sialic acid transferase deficiency in the cells which produce AAT may cause the difference in phenotypic pattern between the two proteins. Such a change in the carbohydrate portion of the AAT molecule could explain other observations made in AAT deficient patients. An alteration in the carbohydrate could produce a protein with a genetically controlled change in electrophoretic microheterogeneity similar to the heterogeneity seen in AAT. Schmid et. al. (12) showed that similar electrophoretic heterogeneity was caused by sialic acid residue in alpha-1-acid glycoprotein. Since some glycoprotein chemists have suggested that the carbohydrate moiety is critical to the transport of proteins from the cells in which they are synthesized (13), and since AAT is synthesized in the liver, a defect in the carbohydrate moiety of AAT might also explain why patients whose AAT has the ZZ phenotype have large quantities of AAT in their livers as determined by fluorescent antibody techniques (14,15).

In addition to the possibility of increasing the understanding of the biochemical defect in AAT deficient patients, these experiments may suggest that AAT deficiency need not be the cause of emphysema in AAT deficient patients. If the genetic defect in these patients is an abnormal glycotransferase, or glycosidase, then other proteins may also have abnormalities in their carbohydrate moieties. The possibility of other defects in addition to AAT deficiency is suggested in a recent publication by Ward and Talamo (16). They demonstrated that patients who have AAT of Pi type ZZ also have a deficiency of an inactivator of chemotactic factor in their serum. At least three interpretations of these experiments are possible: 1) there is an additional genetically controlled protein deficiency in AAT deficiency in AAT deficient patients, 2)

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the inactivator of chemotactic factor is AAT (preliminary studies by Ward and Talamo suggest that the factor is not AAT), 3) AAT may normally inactivate an enzyme which destroys the chemotactic factor inhibitor. If the first of these possibilities is correct, there may be a family of proteins which are abnormal in AAT deficient patients, and the abnormalities may all be caused by the protein which is coded for by the gene which controls the AAT phenotypic expression. For example, if a sugar transferase which was coded for by the defective gene were not specific for AAT a family of proteins with defective carbohydrate chains may exist in these patients. Such a gene could even affect structural glycoproteins such as lung collagen or elastin directly and result in emphysema by this mechanism.

If, however, it can be shown that MM protein differs from ZZ protein because of a point mutation in the structural gene which codes for the primary sequence of AAT, then it would be likely that any defects in AAT deficient patients would be secondary to the deficiency in AAT since with the exception of minor ambiguities in the genetic code (17) one structural gene codes for only one polypeptide.

In summary, AAT in patients with Pi types MM and ZZ differs in two ways, first those with Pi type ZZ have much lower levels of AAT in their blood, and second, AAT manifests complex patterns of electrophoretic microheterogeneity which are characteristic of the genotype. Any postulated mechanism for production of these differences must account for both phenomena. The presence of low levels of antigenically cross-reactive material (CRM) in patients with Pi type ZZ suggests that their genetic defects by the classification of Boyer and his colleagues (18). These investigators infer from structural analyses of human hemoglobin variants and from enzyme variants in microorganisms that most CRM+ mutations involve only one amino acid residue out of hundreds in a typical protein sequence. The difficulty in classifying AAT deficiency in this group of CRM+ defects arises when one tries to define a mechanism for the production of a genetically controlled complex pattern of electrophoretic microheterogeneity as the result of a substitution of a single amino acid.

The success of these experiments is predicated on the knowledge that the gene which causes AAT deficiency also causes, either directly or indirectly, a structural change in the AAT molecule, and the hope that the elucidation of the basis of the structural differences between AAT from individuals of different genotype will suggest the function of the protein coded for by the abnormal gene. If the gene codes for the AAT polypeptide, then standard methods of protein biochemistry may lead to the detection of this abnormality, and may permit interpretation of the nature of the defect in the AAT structural gene. If, however, the gene controls the carbohydrate moiety as in blood group specific glycoproteins, then study of the structure of the carbohydrate moiety may permit insight into the protein which controls the abnormality. These studies will be similar in many ways to studies which elucidated the genetically controlled differences between blood group glycoproteins.

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B). Experimental Design and Procedures: The purpose of this section is to describe the experiments which will provide the data necessary to reject either hypothesis 1 or hypothesis 2 stated earlier. Since all of the patients with Pi ZZ have very low levels of AAT in their blood and almost all of them develop emphysema at an early age, it is likely that some genetically controlled structural characteristic of ZZ protein causes the ATT to occur in the serum in low concentration. The purpose of these experiments is to determine the basis of the structural differences between MM and ZZ glycoproteins.

The structures of glycoproteins are probably determined by at least two genes (19). One of the genes determines the primary sequence of the amino acids in the polypeptide chain whereas the others are thought to control the heterosaccharide structure through enzymes which function as sugar transferases of variable specificity. While the microheterogeneity of some proteins and glycoproteins has been related to variation in the number of charged inorganic groups, desamido forms, or acetylated residues (20), addition of these groups has not yet been shown to be under genetic control. Therefore, there are three likely possibilities to be investigated: 1) the differences in electrophoretic microheterogeneity between MM and ZZ protein are related only to differences in the protein moiety, 2) they are related only to differences in the carbohydrate moiety, or 3) they are related to difference in both protein and carbohydrate moieties.

Possibility 1: If MM protein can be shown to differ from ZZ protein only by one amino acid and if the amino acid substitution can be related to a substitution of a single base in the genetic code (a point mutation), then Hypothesis 1 would be proven, unless ambiguity in the mammalian genetic code can be shown (21, 22). In addition, if MM and ZZ proteins differ in their polypeptide structure, other genetic mechanisms such as gene duplications, gene deletions, etc. or the possibility that heterogeneity is caused by the action of proteolytic enzymes on precursors forms will be considered.

Possibility 2: If, alternatively, MM protein can be shown to differ from ZZ protein by specific types or amounts of carbohydrate moieties only, then it can be suggested that a specific glycotransferase or glycosidase differs in the cells which synthesize different phenotypes. To prove Hypothesis 2 it would be necessary to demonstrate the alteration in the glycotransferase or glycosidase activity in tissues which synthesize AAT.

Possibility 3: If both carbohydrate and protein moieties were abnormal, the nature of the structural differences might suggest a mechanism for production of the differences. For example, if the primary sequence of amino acids in AAT showed an alteration in an amino acid residue which was the site of attachment of a carbohydrate chain, then the differences between phenotypes might still be related to a point mutation in the gene coding for the primary sequence of amino acids of AAT.

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The two hypotheses cannot be resolved by simply removing the carbohydrate from AAT and analyzing the polypeptide and carbohydrate moieties to determine the structural basis for the differences between MM and ZZ proteins. This direct approach to differentiating the two alternative hypotheses is not a practical approach because the methods for removing carbohydrates from proteins are poor and suffer from major inadequacies and complicating side reactions, and because there are no simple and well defined methods for sequencing heterosaccharides. Finally, the structural basis for the microheterogeneity within a single phenotype may have to be resolved before the structural basis of the genetically determined differences between different phenotypes can be determined. The approaches necessary to circumvent these problems and some of the factors which are expected to complicate the experiments are outlined in the Methods section.

General Methods: Enzyme assays: enzyme reaction rates will be measured with a Radiometer pH stat by a potentiometric method when nonchromogenic substrates are employed or with a kinetic recording spectrophotometer when chromogenic substrates are employed. Active sites of trypsin and chymotrypsin will be titrated with p-nitrophenyl-p-quinido benzoate HCl by the method of Chase and Shaw (23). Other enzymes used in analysis will be titrated for active sites when reliable active site titrants are available. Concentrations of enzymes for which no active site titrants are available will be determined by their absorbance at 280 nm.

Since trypsin and chymotrypsin are stoichiometrically inhibited by equimolar concentration of AAT (1) concentration of active sites will be determined by titration with these two enzymes.

Purification of AAT: AAT of MM phenotype will be purified by the method of Cohen and Fallat (24). This method yields sufficient AAT to perform functional studies (25,26). In addition, this is the only published method which yields AAT which is phenotypically unchanged from AAT in whole serum (21). This characteristic is necessary to the performance of these studies.

Schema for analyzing the genetically controlled structural differences between MM protein and ZZ protein:

Problem I: Purification of phenotypically unaltered of ZZ phenotypes.

Methods will be developed to prepare phenotypically unchanged AAT of ZZ phenotype. Several methods will be tried: 1) Method of Cohen and Fallat (24) for preparing AAT of MM phenotype, 2) Method of Crawford for preparing AAT from normal serum, (the integrity of the phenotype has been suggested but not documented), 3) antibody affinity columns eluted with thiocyanate (27) (Hydrogen ion elution will not be used because of the pH lability of AAT), 4) Enzyme affinity columns. (While such columns have been unsuccessful when trypsin and chymotrypsin were used, our recent investigations of the interaction of

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AAT with elastase suggests that the dissociation constant of AAT and elastase is higher than the dissociation constant of AAT with trypsin and chymotrypsin (26), therefore elution of AAT with a strong inhibitor of elastase such as paraoxon will be tried).

Problem II: Determining the number of different polypeptide chains among the components of a single phenotype.

If each of the component peaks of AAT in a single phenotype from one individual represents a different polypeptide chain, then the biochemical differences between MM and ZZ proteins must be analyzed on components from each phenotype which have the same polypeptide structure. Therefore, Problem II is to determine how many different polypeptide chains there are among the components of a single phenotype.

A partial resolution to this problem may be accomplished by end group analysis of phenotypically unchanged, purified MM and ZZ proteins by the dansylation method (28). Crawford (5) determined that the only end group in his preparation of MM protein was a glutamic residue. Isoelectric focusing of the purified protein resulted in a pattern which resembled the MM phenotype but the observation was not confirmed by the standard method of Fagerhol (18). Two other types of data suggest that the components of a single phenotype share the same polypeptide chain. All of the protein in purified AAT is active in the inhibition of trypsin and chymotrypsin (24) and each of the components share similar antigenic determinants with each other and with components of other phenotypes (8).

Further resolution of Problem II can be achieved by purifying the components of a single phenotype and demonstrating that the amino acid analysis of each component is identical to that of the other components within the error of the method which is about 3%. Since Crawford has shown that a pattern similar to the phenotype heterogeneity can be developed with analytic isoelectric focusing of purified MM protein it is possible that the components can be purified by preparative isoelectric focusing.

Problem III: Removing carbohydrate side chains.

Attempts will be made to eliminate the differences between MM and ZZ proteins by digesting the purified AAT of each phenotype with enzymes which only cleave carbohydrates or amino acid-carbohydrate bonds. If differences between MM and ZZ proteins can be eliminated by attacking the carbohydrate only, then two further possibilities must be differentiated: 1) there is an abnormality in heterosaccharide structure only, or 2) there is an abnormality in a serine, threonine, or asparagine residue at a point of attachment of a carbohydrate chain in AAT. There are at least three advantages in eliminating the differences between MM and ZZ phenotypes with enzymes which act on the

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carbohydrate moiety only. First, an abnormality in only one of the three carbohydrate binding amino acids could account for the differences between MM and ZZ proteins. Second, if a defect in the primary sequence of AAT can be ruled out, then the type of enzyme used to eliminate the differences between MM and ZZ protein may suggest the function of the abnormal protein which is coded for by the gene which controls the AAT phenotypic expression. For example, if the differences are eliminated by neuraminidase, then the abnormal protein in AAT deficient patients is likely to be a sialic acid transferase. Finally, if the differences between MM and ZZ proteins are eliminated by an enzyme which cleaves carbohydrate chains, then an analysis of the differences between the chains cleaved from MM and ZZ proteins may suggest the function of the abnormal protein coded for by the AAT gene.

Two types of sugar cleaving enzymes are of particular interest, neuraminidase and N-acetyl- α -D-Galactosaminidase. Neuraminidase, an enzyme which cleaves terminal sialic acid residues from the carbohydrate chains, is important because Bell and Carrell (11) have claimed to change an AAT protein with MM phenotype as determined by Fagerhol's method (8) into a protein with ZZ phenotype by partial digestion with neuraminidase. These investigators have not ruled out the possibility that the modified MM may resemble ZZ only superficially. Further proof would consist of showing that complete digestion of MM and ZZ proteins with neuraminidase would yield AAT which still identical in acid starch gel.

Since there is some evidence to suggest that an attack on sialic acid residues will obliterate phenotypic differences between MM and ZZ proteins, this experiment will be performed first. Ideally, in order to demonstrate that phenotypic differences between MM and ZZ protein are due to the carbohydrate moiety of AAT, the carbohydrate moiety should be totally removed and the phenotypes should then be re-examined. However, cleavages of the carbohydrate from proteins is a complicated task. Carbohydrates are attached to mammalian glycoprotein by three main types of bonds. In the glycosylamine linkage, involving the amide group of asparagine so far only N-acetyl-glucosamine has been found to be the glycosyl connecting unit (Gly-Nac-Asn). The amino sugar linked O-glycosidically to peptide-bonded serine or threonine residues in glycoproteins seems to be invariably N-acetyl-galactosamine (29). The Gly-Nac-As bond is resistant to enzymes in whole glycoproteins and to mild acid and alkaline hydrolysis procedures but the O-glycosidic linkage involving the hydroxyl groups of serine and threonine can be cleaved in most glycoproteins and to mild acid and alkaline hydrolysis procedures but the O-glycosidic linkage involving the hydroxyl groups of serine and threonine can be cleaved in most glycoproteins with the enzyme N-acetyl- α -D-Galactosaminidase, after the glycoprotein is predigested with neuraminidase (30). Therefore, if the conclusions of Bell and Carrell (11) cannot be verified as outlined above, digestion serially with neuraminidase and N-acetyl- α -D-Galactosamine will be carried out on MM and ZZ proteins. The phenotypes

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will then be re-examined to determine if the differences have been eliminated. If the single or the dual enzyme digestion of MM and ZZ protein can abolish their differences, then experiments will continue with Problem IV.

Problem IV: Differentiation of a primary defect in carbohydrate structure from a defect in carbohydrate structure caused by a defection in amino acid sequence in AAT.

To determine if an amino acid defect exists in MM and ZZ proteins, the respective proteins will be cleaved into peptides. The peptides will be separated by column chromatographic methods, and amino acid analysis will be carried out on the small peptides (see appendix). Special attention will be given to carbohydrate containing peptides and the amino acid-carbohydrate linkages will be determined. If MM and ZZ differ by a single small peptide, isolation and sequencing of the peptide will be attempted by the Edman degradation method (31).

If only the carbohydrate moieties are abnormal and if the abnormality suggests a defect in a specific sugar transferase in the cells which synthesize AAT, then the absence of such a sugar transferase will be proven by examination of tissues from subjects with AAT phenotypes MM and ZZ. For example, if the difference between MM and ZZ proteins is determined only by terminal sialic acid residues as suggested by Bell and Carrell, then a sialic transferase will be sought in tissues from subjects with AAT phenotypes MM and ZZ. The experiments will be carried out using experimental designs which are similar to those of Zideman. They showed that the genetically controlled differences between types A and B blood group substances were determined by difference in sugar transferases in certain tissues. Tissues were tested for glycosyl transferases of appropriate specificity. Both low molecular weight oligosaccharides of known structure and macromolecular glyco-proteins were used as sugar acceptors; nucleotide diphosphate sugars used as sugar donors and the monosaccharide component carried the radioactive marked. Finally, it may be possible to convert one phenotype to another with the appropriate sugar transferase, sugar moiety, and sugar donor.

Problem V: Analysis of the biochemical basis for phenotypic differences between MM and ZZ proteins if the differences cannot be eliminated by removing sugar residues.

If the differences between MM and ZZ proteins cannot be eliminated by glycosidases or glycosaminidases then the problem of defining the differences between these proteins is more complicated. The carbohydrate chains which are different will likely be difficult to separate in tact from the protein moieties, and the possible amino acid defects in the primary sequence of AAT are not limited to those amino acids which serve as attachment points for the carbohydrate chains.

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AAT will be cleaved at methionyl residues with CNBr (35). Then in order to facilitate further digestion of AAT into peptides, disulfide bonds will be reduced with dithiothreitol by the method of Cleland (36) and S-carboxy methylated (37). At each step peptide fragments will be separated from each other and the carbohydrate containing peptides will be separated from the non-carbohydrate containing peptides. Enzymatic digestion will be carried out on neuraminidase treated peptides to facilitate complete digestion (38). Enzymes which may be used include trypsin, chymotrypsin, papain, subtilisin, etc. Those peptides of both classes which are identical between MM and ZZ proteins will not be further analyzed. Carbohydrate free peptides from MM protein will be separated by ion exchange chromatography (39), analyzed for AA content, and matched with peptides in ZZ proteins for differences (see appendix). Particular attention will be paid to single amino acid substitutions which could be caused by the AAT controlling gene.

In order to analyze the carbohydrate containing peptides first the peptide moiety will be separated from the carbohydrate moiety so that the polypeptide can be analyzed by the methods described in the preceding paragraph. Removal of the carbohydrate chains from seryl or threonyl bonds will probably be achievable with N-acetyl- α -D-Galactosaminidase (40) or by alkaline hydrolysis (41). However, removing in-tact heterosaccharide chains from the aspartyl bond is more troublesome. Partial acid hydrolysis may have some success (42), however, this treatment may also cleave some peptide bonds.

For isolation of the carbohydrate chains, the peptide will be digested away as completely as possible with proteolytic enzymes. Carbohydrates will be separated from peptides and free amino acids on ion exchange columns (43) or on concanavalin columns (44). The connecting amino acids will be cleaved with the enzymes, 4'-L-aspartyl glycosylamine amido hydrolase or acetyl- α -D-Galactosaminidase (see appendix) and the connecting amino acid identified. Further clarification of differences in carbohydrate chains between MM and ZZ proteins poses a problem which cannot be solved in advance since there are no reliable methods for sequence determination of these chains. Some success has been achieved with a combination of gentle acid hydrolysis and specific glycosidases, but the methods are not universally applicable (45). If the differences between MM and ZZ proteins can be localized to the carbohydrate moieties only then another possible approach would be to examine tissue from ZZ patients for absences of all the specific sugar transferases which are involved with synthesis of AAT. Since AAT contains only hexoses, hexosamines, acetylneuraminic acid, and fucose (46), seeking transferases for each of these sugars may be a manageable experiment.

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Appendix 1

Hydrolysis of glycoproteins and amino acid analysis: In order to reduce the loss of cystine, methionine and tyrosine and artifact information due to oxidation of amino acids, amino acid hydrolysis will be carried out by the method of Blackburn (47). The protein will be dissolved in 6N HCl in a pyrex container, frozen, then the glass container will be evacuated to below 0.05 mm of Hg. The protein solution will then be thawed for degassing, re-frozen, and sealed. To reduce the interaction of carbohydrate degradation byproducts solutions of glycopeptide will be kept below 0.1%. Samples will be heated in an oven at 110°C for 24, 48, and 72 hour periods, the top of the tube removed and the tube will be evaporated to dryness. Before analysis the residue will be dissolved in water and brought to pH 6.5 with phosphate buffer and allowed to stand for 4 hours to allow oxidation of cysteine to cystine. It will then be prepared for application to the amino acid analyzer.

Analysis of amino acids will be performed on a Beckman HP 119 amino acid analyzer equipped with an Autolab integrator using Beckman Spinco AA-15 resin at a constant temperature of 52°C. Tryptophan will be determined by hydrolysis in p-Toluenesulfonic acid by the method of Liu (49). Methionine will be determined by oxidizing the peptide to methionine sulphoxide by the method of Stegeman (50) before analysis. Values for serine and threonine will be extrapolated to zero time from the hydrolyzed heated for 24, 48, 72 hours. Values for isoleucine and valine will be determined by hydrolysis for 72 hours. Cystine will be determined on separate peptide samples as cysteic acid after performic acid oxidation (51).

Appendix 2

Preparation of peptides for mapping: Denatured, reduced and carboxy-methylated MM or ZZ proteins will be cleaved first by cyanogen bromide (35), and the purified fragments will be digested with trypsin (52). The peptides obtained at each step will be isolated by gel filtration or ion exchange chromatography. The theoretical yield of peptides from the procedures would be 46. At both steps, attempts will be made to isolate TCA insoluble peptides by precipitation with 5% TCA. If TCA insoluble peptides are found, further cleavage of peptides may be required by other proteolytic enzymes before they are further analyzed.

Initial separation of peptides will be carried out with anion exchange chromatography with Dowex 1-x2, using the method of Fantasu (53). The peptides will be eluted with a gradient starting with a buffer which is 1% collidine, 1% pyridine, and 6.25 mM in acetic acid (pH=8.2) and progressive drops in Ph until that of 5N acetic acid. Peptides will be further separated on a cation exchange column mounted on the amino acid analyzer using Beckman-Spinco resin PA-35. The chromatogram will be developed with a Buchler varigrad

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gradient maker which will employ a starting buffer of 0.2M pyridine acetate buffer at pH=3.1, and a second buffer of 2.0M pyridine acetate buffer at pH=5.0 as described by Jones (39). These methods are first approximation and final methods are first approximation and final methods will be determined by experimentation.

Appendix 3

Purification of alpha-D-galactosaminidase: Alpha-D-galactosaminidase will be purified from beef liver by the method of Weissman and Hinrichsen (40). These investigations achieved a 700 fold purification of the enzyme using ammonium sulfate fractionation, column chromatography on DEAE-cellulose, and Sephadex G-150 sequentially.

Appendix 4

Purification of 4'-L-Aspartylglycosylamine amido hydrolase (AAH): Purification of this enzyme will be performed by the method of Makino (54), from hog serum. The methods used sequentially, ammonium sulphate precipitation, CM-cellulose chromatography, DEAE-cellulose chromatography, and calcium phosphate chromatography. The material recovered is still heterogeneous, but it contains no proteolytic or glycosidic activities toward glycoproteins.

Appendix 5

Carbohydrate analysis: The procedure to remove carbohydrate from seryl and threonyl residues by alkaline hydrolysis or enzymes and from aspartyl residues by alkaline hydrolysis or enzymes and from aspartyl residues by acid hydrolysis and the procedure to separate the free carbohydrate chains have been referenced in the text (40,41,42,43). Analysis of the carbohydrate chains will be performed by gas-liquid chromatography after methanolysis by the methods of Clamp, Bhatti and Chambers (55).

Preliminary experiments will include the preparation and analysis of carbohydrate chains from glycoproteins of known structure in order to verify the method. Samples of suitable weight will be combined with sufficient internal standard to give peaks of similar height. 0.5 ml of 1.5N methanolic HCl will be added to each dried ampoule. The ampoules will be sealed under nitrogen and heated at 90°C for 24 hours or varying times and temperatures when indicated. After cooling and neutralization re-N-acetylation will be carried out by addition of acetic anhydride. Supernatants will be pooled and acetic anhydride removed with rotary evaporation at 25°C. The trimethylsilyl derivatives will be prepared by adding 50 ul of TriSil-Z to the dried material in a screw capped vial. The vial will immediately be sealed and the reaction will be allowed to proceed for 30 minutes at 25°C. A 2 ul aliquot of the

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sample will be injected into the GLC. The conditions of chromatography are described by Clamp, Bhatti, and Chamber (55). Conditions of hydrolysis, re-N-acetylation, and trimethylsilylation will be varied to give maximal yields.

Appendix 6

Unpaid Consultant: Since the experience in this laboratory with carbohydrate analysis in glycoproteins is limited, consultative services have been arranged with Dr. Johnathon Doxon, Ph.D. who is a glycoprotein chemist in the Hormone Research Laboratory and who is actively performing carbohydrate analyses on glycoproteins with gas-liquid chromatography.

C). Significance of this work: Since the occurrence of AAT deficiency in some patients with emphysema is one of the few clues available to the nature of the biochemical processes in human emphysema, an understanding of these processes may lead to modes of therapeutic intervention. This section will attempt to answer two questions: first, what is the importance of AAT deficiency to emphysema from all causes in this country, and second what contribution will the answers to the questions posed in this protocol make to the understanding, treatment and control of AAT deficiency.

Two questions must be answered in order to determine the importance of AAT deficiency to human emphysema. First, what proportion of the population with abnormal AAT genes get emphysema as a result of these genes, and second is there a link between the the AAT related emphysema and the more common form of emphysema seen in smokers? The answer to the first question is controversial. While all investigators agree that only a rare patient with pi type ZZ survives to the age of 50 without emphysema (7). Estimates of the incidence of lung disease in subjects with pi type MZ vary from nil in Eriksson's study to substantial in the work of Lieberman (56) and of Kueppers and Larson (57), who estimated that 26 and 29% respectively of the patients with chronic obstructive pulmonary disease had AAT with pi types ZZ or MZ. The incidence of the Z gene in Americans has been estimated from 0.01 to 0.016 (9, 57). In addition, if Eriksson's estimate of the prevalence of patients with pi type ZZ of 0.00057 (7) is also found in Americans, then more than 140,000 Americans are at high risk of developing emphysema related to AAT deficiency. In addition, the data of Black and his colleagues (58) suggests that nonsmoking homozygotes may get the emphysema about a decade later than smoking homozygotes. Therefore, smoking may exacerbate the propensity of subjects with pi type ZZ to get emphysema or perhaps the two forms of emphysema may be produced through a common biochemical mechanism. One possible common pathway was suggested by Cohen (4). If there is a common pathway, then drugs developed to prevent

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emphysema in AAT deficiency may also prevent emphysema in smokers who get emphysema.

Evidence was reviewed in the introduction to this protocol which shows that most investigators who study AAT deficiency work on the premise that the low levels of AAT are the proximate cause of the emphysema in deficient patients, and that the emphysema is caused by the unrestrained action of one or more enzymes on pulmonary tissues. This protocol presents evidence which suggests that defects other than the AAT deficiency are present in these patients and suggests experiments which might either show that these other defects are secondary to the deficiency in AAT or show how to determine the biochemical nature of the other defects if the other defects are not secondary. The possibility that other defects occur in the same people and that one of the other defects may cause the emphysema which these people develop must be eliminated before investigations in this field become completely confined to AAT.

D). Probable Future Direction of this Work: If the weight of evidence suggests that alpha-1-antitrypsin is the protein coded for by the abnormal gene in deficient patients, then it would be likely that all of the other biochemical lesions would be secondary to this lesion. This information would suggest that addition of alpha-1-antitrypsin-like activity to the blood of deficient patients might prevent the emphysema which they develop. Therefore, methods for accomplishing this goal would be investigated. The goal might become possible through several possible routes. Our SCOR B-1 research on the biochemical mechanism of the active sites of alpha-1-antitrypsin may permit the design of drugs which would replace the function of the missing inhibitor. Other lines of investigation in the proposed research may suggest methods of releasing alpha-1-antitrypsin from livers of deficient patients. In addition, many other investigators are exploring other means for accomplishing these ends.

If, however, this research suggests that biochemical lesions other than the low levels of alpha-1-antitrypsin may exist in these patients then future research would explore these other lesions to determine if one of them might be the cause of emphysema in alpha-1-antitrypsin deficient patients.

E). Relationship to Dr. Cohen's SCOR Project: Dr. Cohen's SCOR project is related to the determination of the mechanism of action of the inhibitory sites on AAT and this protocol is related to the determination of the genetic differences between AAT of pi types MM and ZZ. Since patients with pi type ZZ have trypsin inhibitory capacities commensurate with their levels of AAT,

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the active site of AAT is probably functional in ZZ protein. Therefore, it is unlikely that experiments in one protocol will be useful in the goals of the other protocol. In fact, the procedures to be employed in this protocol will very likely destroy the inhibitory site on AAT. Therefore, there will be little if any overlap between the protocols.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1974

Grant application No. 962

CHRONIC PULMONARY DISEASE

To: The committee comprising Drs. Liebow, Sommers and Wyatt

Subject: David W. Cugell, M.D., Northwestern University, Evanston
New application No. 962
"Tests of small airway function as screening procedures for
obstructive lung disease"

History

A preliminary inquiry was handled as case No. 196, and the Executive Committee voted to encourage formal proposal.

Request

Application No. 962 requests \$26,857 plus two additional years. Total duration of the study is estimated at six years.

Documents Submitted (Attached)

1. Application dated 1/28/74 (13 pages).
2. Letter dated January 28, 1974 from Jeremiah Stamler, M.D. concerning human subjects for the study, and endorsing the proposal.

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F.W.N.

Enclosures

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

FEB 6 1974

Date: 1/28/74

1. Principal Investigator (give title and degrees):

David W. Cugell, M.D.
Bazley Professor of Pulmonary Diseases
Chief, Pulmonary Section, Department of Medicine

2. Institution & address:

~~Northwestern University Medical School~~
~~303 East Chicago Avenue~~ 619 Clark Street
~~Chicago, Illinois 60611~~ Evanston, Illinois 60201

3. Department(s) where research will be done or collaboration provided:

Medicine; Community Health and Preventive Medicine - The Medical School
303 East Chicago Avenue
Chicago, Illinois 60611

4. Short title of study:

Tests of small airway function as screening procedures
for obstructive lung disease

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 6 years (three years with this application)

7. Brief description of specific research aims:

Several new lung function testing procedures have been widely acclaimed because of their purported value for early detection of chronic obstructive pulmonary disease (COPD). These tests have great potential usefulness for screening purposes, but rigorous proof of their predictive value is not now available. A prospective study will be undertaken in a large group of coronary-prone men already enrolled in a long term program of medical intervention with respect to diet, blood pressure control and smoking cessation. The major objectives of the pulmonary studies that will be conducted as a part of the periodic evaluation of these patients are:

1. To determine the relative value and significance of measurements of:
(a) closing volume and closing capacity, (b) expiratory flow at 50 per cent of the vital capacity and smaller lung volumes, and (c) traditional indices of expiratory airflow such as the maximum mid-expiratory flow (MMF) and the forced vital capacity in one second (FEV₁).

2. Measurement of the relative changes with time in the above tests.

3. Does intervention - specifically cessation of smoking - alter the changes with time in these tests?

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8. Brief Statement of Working Hypothesis:

A. Introduction. The initial structural and functional abnormalities in COPD - prior to the appearance of overt clinical manifestations - occurs in small airways with a diameter of two mm or less. Traditional lung function tests reflect the status of the larger airways only, and thus may be quite normal despite the presence of disease of the small airways. Several tests, some ideally suited to large scale population surveys, are now available to assess the status of these small airways. What value they may have for this purpose can only be determined by carefully controlled, longitudinal study of a cooperative, stable population.

B. Small airway function. The initial event in the prolonged, irreversible sequence that terminates with the clinical picture and functional impairment of COPD is thought to be inflammatory changes limited to the small (less than 2 mm in diameter) airways (Hogg, New Engl. J. Med. 258:1355, 1968). If so, it is of considerable importance to develop methods for assessing small airway function because patients at this incipient stage of COPD are essentially asymptomatic and the process may be reversible. Traditional ventilatory function tests are not sufficiently sensitive to detect COPD at this early stage because the standard tests primarily indicate the flow resistive properties of the large airways. To assess the properties of the small airways special procedures must be employed such as: (1) measurement of pulmonary mechanics or gas distribution at several different breathing frequencies, (2) determination of the airway closing volume or capacity, and (3) measurement of expiratory airflow at low lung volumes. To effect any significant reduction in the incidence of COPD, patients destined to develop the disease must be identified when they are entirely asymptomatic and, it is hoped, in a reversible phase of their illness. Once symptoms are present, and traditional lung function tests become abnormal, the disease is well established and irreversible.

Apart from the specificity, sensitivity, reproducibility, and clinical usefulness of the above three categories of tests of small airway function there is the practical matter of their suitability for large-scale field use. Thus, some trade off may be necessary between tests that are highly specific but cumbersome, uncomfortable, time-consuming, and expensive; and other tests that are less specific but simple, painless, rapid and inexpensive. Because it is necessary to swallow an esophageal balloon, the measurement of lung mechanics at different breathing frequencies cannot be used as a screening test.

The closing volume or closing capacity and expiratory flow at low lung volumes have been acclaimed as being indicative of the status of the small airways and technically suitable for use in screening programs for respiratory health. Whether the closing volume or closing capacity is superior to flow measurements for early detection of minimal disease is uncertain. In nine patients with abnormal alveolar-arterial oxygen gradients and frequency dependence of compliance, Gelb and Zamel (New Eng. J. Med. 288:395, 1973) found abnormal expiratory flows in only seven. On the other hand, abnormal values for the closing volume were found in 72 percent of 46 smokers whereas one or more conventional tests were abnormal in only 30 percent (McCarthy, Amer. J. Med. 52:747, 1972). We have surveyed 275 non-smoking employees of a Seventh Day Adventist Hospital and compared the closing volume, forced expired volume in one second (FEV₁), and flow at 25 percent vital capacity (Solliaday, Amer. Rev. Resp. Dis. 107:1107, 1973). The closing volume was the only test to show

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8. Brief Statement of Working Hypothesis: (continued)

a significantly different response between employees with respiratory symptoms and completely asymptomatic individuals. The closing capacity appears to be slightly more sensitive than the closing volume in discriminating early dysfunction (Buist, Amer. Rev. Resp. Dis. 107:735, 1973). Whether small airway dysfunction necessarily means the eventual development of overt disease remains to be determined. All published normal values for the closing volume or capacity in adults indicates a progressive increase with age. Whether the change with time is different in patients with normal values at the outset than in patients with abnormal values will require some years of repetitive observations to determine.

C. Large airway function. Contrary to the relative novelty of tests for measurement of small airway function are the well established procedures for assessing large airways. The FEV₁ and maximum mid-expiratory flow (MEF) have been in routine use for many years, and there is a large body of accumulated, long-term experience with them. Studies of the same population group, repeated at intervals for some years, have been published so that the changes with time are known. Similar studies in patients with established obstructive lung disease indicate an exponential decline in FEV₁ (Chandrasekhar, Morse, Nam and Cugell, Amer. Rev. Resp. Dis., submitted for publication).

D. Temporal changes in ventilatory function. Normal values for lung function tests are imprecise, and it is traditional to allow for a twenty percent reduction below the predicted normal value before considering a specific result definitely abnormal. In contrast to these wide variations in predicted values, individual patient performances are highly reproducible. If the same person repeats the same maneuver at specified intervals over a period of time, a performance pattern can be established against which that person's subsequent results can be compared. Thus, repetitive measurements at yearly intervals will establish a regression line for that test against age for that person. Deviation from the regression line will provide much greater sensitivity in identifying an abnormal result than comparison of an isolated measurement with a number calculated from a prediction formula.

E. Mass screening. Screening tests can be evaluated according to several characteristics (Chamberlain, "Recent Advances in Medicine," 1973) such as sensitivity, specificity, predictive value and reproducibility. Safety, patient acceptability, economy and convenience are additional important features. The closing volume or capacity, and expiratory flow measurements are completely safe, acceptable to the patient, inexpensive and convenient but other characteristics have not yet been defined. The predictive value of these, or any other, screening procedures are of critical importance. In order to determine if an abnormal result signifies definite disease, some independent means of disease identification must be employed. Since the onset of chronic obstructive pulmonary disease is uncertain and the clinical course highly variable, there is considerable difficulty in identifying patients with COPD at an early, beginning phase of their illness. On the other hand, once established, COPD is easily recognized from the clinical symptoms and limitation of expiratory flow as measured by the FEV₁ and MMF. Thus, the predictive value of closing volume or capacity and low lung volume flow rates cannot be ascertained without careful, long-term observation of a cooperative group, some of whom are destined to develop COPD.

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8. Brief Statement of Working Hypothesis: (continued)

The reproducibility of a test on repeated measurements in the same individual is affected by biological factors and a variety of objective and subjective measurement errors. We found that observer variation was minimal for good quality closing volume tracings (Morse, Clin. Res. 20: 759, 1972). Our own comparison of the relative reproducibility of expiratory flow at low lung volumes and closing volume or capacity has not been completed.

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(See appended pages)

9. Details of experimental design and procedures (append extra pages as necessary)

A. Introduction. A major difficulty in mounting an effective epidemiological study is developing case finding methods, procedures for follow-up and training personnel to conduct these essential tasks. At this Medical Center there now exists a unique opportunity to utilize a patient population now being enrolled in prospective intervention trials. This nationwide, cooperative investigation into coronary artery disease is entitled, "Multiple Risk Factor Intervention in Coronary Prone Individuals" and is supported by the National Heart and Lung Institute. The annual project costs are several million dollars nationally with several hundred thousands of that total allocated for the three centers in the Chicago area. Approximately fifty thousand men aged 35-57 will be screened in this region; at least nine hundred with a high-risk of developing myocardial infarction will be selected and evaluated at frequent intervals. These high risk patients will be enrolled in various programs to determine if the expected incidence of myocardial infarction can be reduced. One group will be enrolled in a program that includes dietary modification, treatment of high blood pressure, and advice to help the patient quit smoking; another group will be referred to their private physicians for management. This project will be conducted in three phases: (1) identification of patients at risk based upon an elevated blood pressure, cigarette use and elevated blood cholesterol, (2) detailed study of those deemed to be at increased risk and (3) intervention by one means or another. The first phase - identification of suitable patients - is now under way. The intensive study or second phase will include a detailed clinical evaluation, blood chemistry profile, and rest and exercise electrocardiography and vectorcardiography. These intensive studies will be conducted at St. Joseph's Hospital, a community hospital nearby the Northwestern University McGaw Medical Center. By performing a few additional tests that will require no more than 15 additional minutes, the objectives of this proposed project can be fulfilled. Because of the established organizational structure and system for data collection and follow-up, there is a unique opportunity for additional epidemiological investigation - providing the extra procedures do not impose any undue burden on the patient or interfere with the primary project. Any additional studies that can be included will provide information at a fraction of the cost that would be required if an independent program for locating patients, defining their health status and obtaining follow-up observations was required.

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(continued)

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9. Details of Experimental Design and Procedures: (continued)

B. Selection of patients. All of the high risk men will be evaluated in the St. Joseph's Hospital laboratory and will be subjected to the respiratory maneuvers required for the purposes of this project. All 900 men will be studied at the time they enroll in the program, and annually thereafter. One sixth, or 150 men, will receive their treatment at the St. Joseph's Hospital center, and they will be studied at four month intervals. A careful evaluation of each person's respiratory status will be performed, including a physical examination and completion of the detailed respiratory questionnaire described in Report of Workshop on Epidemiology of Respiratory Disease published by the National Heart and Lung Institute, 1971. All of the clinical and laboratory results will be co-mingled with the data obtained as part of the primary project. Insofar as possible, the intervention program the patient may be following, particularly with regard to his smoking habits, will not be known to the technician and will remain unknown to those analyzing the results.

C. Test procedures. The patients will perform three rapid, forced expiratory efforts and three leisurely expirations. The forced expiratory effort will be recorded such that the peak or maximum expiratory velocity and the airflow at various selected points over the range of vital capacity will also be available. The superimposition of timing signals, triggered by the rapid rise of expiratory velocity, will permit recovery of the forced expired volume at several time intervals including the customary one second value. Thus, a single forced expiratory effort will provide information relevant to airflow dynamics at low lung volumes and the traditional measures of expiratory flow such as the FEV_1 , MMF, peak expiratory flow etc. The same recording apparatus will be used to inscribe the expired volume during a more leisurely expiration for calculation of closing volume and closing capacity, with the requisite nitrogen concentration recorded on the axis previously used for expiratory velocity. In addition, the slope of the "alveolar plateau" will be calculated. As yet, it is not clear which of these three calculations from this simple maneuver is most meaningful. Calculation of the closing capacity is unduly time-consuming when performed manually. A digital integration of the expired nitrogen concentration would provide a convenient method for calculating the mean alveolar nitrogen concentration and greatly simplify the closing capacity computation. Inasmuch as almost all of the patients will be gainfully employed and relatively well, no procedural difficulties are envisioned, and it seems likely that all of the tests can be completed within twelve to fifteen minutes. It is uncertain whether three repeat efforts for each procedure will be required, but it is appropriate to establish a fixed procedure, particularly at the outset. Fewer efforts may suffice at the time of the repeat examination. All patients will have a blood sample assayed for alpha-1-antitrypsin levels in order to define those within the population under study with a partial or complete enzyme deficiency. This will be done at the time of the initial evaluation and not repeated subsequently.

D. Equipment. In order to minimize the technician time, provide maximum convenience for the operator and minimum time for the patient, semi-automatic apparatus will be used. Such a system can be provided by Systems Research Laboratories, Inc. An appropriate combination of flow detection devices, gas analyzers and digital computing units will enable the operator to perform all of the required tests at one sitting within twelve to fifteen minutes. The apparatus will require four to five square feet of floor space. There will be a graphic record of each test as well as a digital display of the results. Prior to its use the equipment will be subjected to rigorous verification of its accuracy, reproducibility and stability plus frequent checks with mechanical standards and duplicate determination on staff members using equivalent apparatus at the Northwestern University McGaw Medical Center Pulmonary Function laboratories. A preliminary scheme for the semi-automatic calculation of mean alveolar nitrogen concentration for the closing capacity computation is included in an appendix.

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9. Details of Experimental Design and Procedures: (continued)

E. Evaluation of results. The design of this multiple risk factor intervention study will provide definitive information about the effects of diet, aging, blood pressure control and alteration of smoking habits on selected lung function tests. Traditional tests such as the FEV₁, MMF and peak expiratory flow - which are primarily dependent upon flow resistance in the large airways - will be compared with the closing volume or capacity and flow at low lung volumes which reflect small airway function. This comparison will provide information on the relative sensitivity of these two classes of lung function tests, rate of change with age, and ability to predict the eventual development of overt COPD. The significance, if any, of tobacco usage will become evident from a comparison of the results between non-smokers, smokers and ex-smokers.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory space has been set aside at St. Joseph's Hospital to conduct the intensive study of all candidates selected for this long-term project. The equipment required for the objectives of the multiple risk factor intervention program have been installed but suitable apparatus for the study of lung function is not now available. Space to accommodate the special self-contained, specially designed equipment mentioned in Section 9 is available. Backup laboratory services in the form of trained technical and professional personnel, comparable equipment and alternative apparatus in the event of equipment breakdown are available from the Pulmonary Function Laboratories of the Northwestern University - McGaw Medical Center. The principal investigator of this proposal is in charge of those laboratories. They are equipped with a full range of gas analyzers, single and multichannel recorders, flow measuring and sensing devices, and some engineering support service.

11. Additional facilities required:

This project can be accommodated entirely in existing facilities.

12. Biographical sketches of investigator(s) and other professional personnel (append): see attached

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
see attached

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12. Biographical Sketch

David W. Cugell, M.D.

BIRTHDATE: REDACTEDBIRTHPLACE: REDUCATION: Deerfield Academy - R
Yale University, B.S. degree - R
Long Island College of Medicine, M.D. degreePOST-GRADUATE TRAINING

Rotating Internship - Bronx Hospital, New York City - 1947-1948
 Medical Intern - Albany Hospital, Albany, New York - 1948-1949
 Medical Assistant Resident - Albany Hospital, Albany, New York - 1949-1950
 Research Fellow - Thorndike Memorial Laboratory, Harvard Medical School,
 Boston City Hospital - 1950-1951
 U. S. Army Medical Department - 1951-1953
 Research Fellow - Thorndike Memorial Laboratory, Harvard Medical School,
 Boston City Hospital - 1953-1955

FELLOWSHIP AWARDS

American Heart Association Research Fellowship - 1953-1955
 American Thoracic Society - Potts Memorial Foundation - 1953-1952
 Career Research Development Award - National Heart Institute, U.S. Public
 Health Service - 1962-1967

SPECIALTY CERTIFICATION

American Board of Internal Medicine - 1955
 Sub-Specialty Board of Pulmonary Diseases - 1956

PROFESSIONAL SOCIETY MEMBERSHIPS AND PROFESSIONAL ACTIVITIES

REDACTED

REDACTED

PRESENT FACULTY POSITION

Bazley Professor of Medicine, Northwestern University Medical School, and Chief,
 Pulmonary Diseases Section
 (Affiliated with Northwestern University Medical School since 1955, from Associate in
 Medicine to Professor to the Bazley Chair; from Director, Pulmonary Function Laboratory,
 to Chief, Pulmonary Section to present Director, Pulmonary Diseases Section, Dept. of Med.)

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13. Publications - Papers

1. Kettel, L. J., Lesage, C.H., Jr., Webster, J.R., Jr. and Cugell, D.W.: Upper Extremity Blood Flow in Patients with Chronic Obstructive Airways Disease, Am. J. Med. Sci., 258:52-58, 1969.
2. Cugell, D.W., "Use of Tape Recordings of Respiratory Sound and Breathing Patterns for Instruction in Pulmonary Auscultation", Am. Rev. Resp. Dis., 104:948-950, 1971.
3. Addington, W.W., Cugell, D.W., Westerhoff, T., Shapiro, B., and Smith, R., "The Pulmonary Edema of Heroin Toxicity--An Example of the Stiff Lung Syndrome", Chest, 62:199-205, 1972.
4. Allen, T.W., Addington, W.W., Rosendal, T., and Cugell, D.W., "Alveolar Carbon Dioxide and Airway Resistance in Patients with Postexercise Bronchospasm", Am. Rev. of Respiratory Disease, Vol. 107, #5:816-821, May, 1973.
5. Gracey, D.R., Kwaan, H.C., and Cugell, D.W., "The Treatment of Pulmonary Embolism", Clinical Conference in Pulmonary Disease, Chest, 63:1106-1011, 1973.

13. Publications - Abstracts

1. Morse, S., Nam, K., Jacobstein, J., and Cugell, D., "Comparison of Single Breath Bolus and N₂ Washout Methods for Measuring Closing Volume", Clin. Res., 20:759, 1972.
2. Chandrasekhar, A.J., Morse, S., Nam, K., and Cugell, D.W., "Limitations of FEV₁ for Assessing the Course of Chronic Obstructive Pulmonary Disease (C.O.P.D.), Clin. Res., 107:1103, 1973.
3. Solliday, N.H., Solomon, N., Morse, S., Nam, K., Gracey, D. and Cugell, D.W.: Airflow and Closing Volume Measurements in Early Chronic Respiratory Disease, Am. Rev. Resp. Dis. 107:1103, 1973.
4. Solomon, D., Solliday, N., Buehler, N. and Cugell, D.W.: Closing Capacity and Flow Rates at Low Lung Volume in Acute Alterations of Bronchomotor Tone. Submitted to Am. Lung Assn.

1003540016

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

David W. Cugell, M. D.

10%

-

Technical

Mary Hansson, R. N.

100%

Fringe Benefits - 11.1%

R

Sub-Total for A

R

B. Consumable supplies (by major categories)

Recording paper, mouthpieces,
test tubes

-

400

Sub-Total for B

400

C. Other expenses (itemize)

Miscellaneous tools, electronic parts

100

Sub-Total for C

100

Running Total of A + B + C

13,832

D. Permanent equipment (itemize)

1. Variable speed, variable stroke
calibration pump - to be constructed
2. Systems Research Laboratory console
with x-y-y recorder, nitrogen meter,
m-10 predictive screener with mid-flow
board, memory plotter and dual range
flow transducer

150

10,800

10,950

Sub-Total for D

2,075

E

E. Indirect costs (15% of A+B+C)

Total request

26,857

15. Estimated future requirements

| | Salaries | Consumable Suppl | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|------------------|----------------|------------------|----------------|--------|
| Year 2 | R | 400 | | | 2,160 | 16,558 |
| Year 3 | | 400 | | | 2,226 | 17,069 |

1003540017

s. 12

16 Other sources of financial support

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--------------------------------|----------------------|------------------------|
| Graduate Training Grant in Pulmonary Diseases | US PHS - NHLI | \$31,000 per year | April, 1966-June, 1975 |
| Bazley Trust | Private | \$40,000 per year | Indefinite |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--------------------------------|----------|---------------------------------|
| Validation of newer methods of detecting early COPD (a short term project based upon bronchospasm provocation) | Chicago Lung Assn. * | \$12,641 | July 1, 1974 - June 30, 1975 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Principal investigator:

Typed Name David W. Cugell, M.D.Signature David W. Cugell Date 1/28/74Telephone R
Area Code Number Extension

Checks payable to

Arthur T. Schmeeling, Vice Pres. & Controller

Mailing address for checks

633 Clark StreetEvanston, Illinois 60201

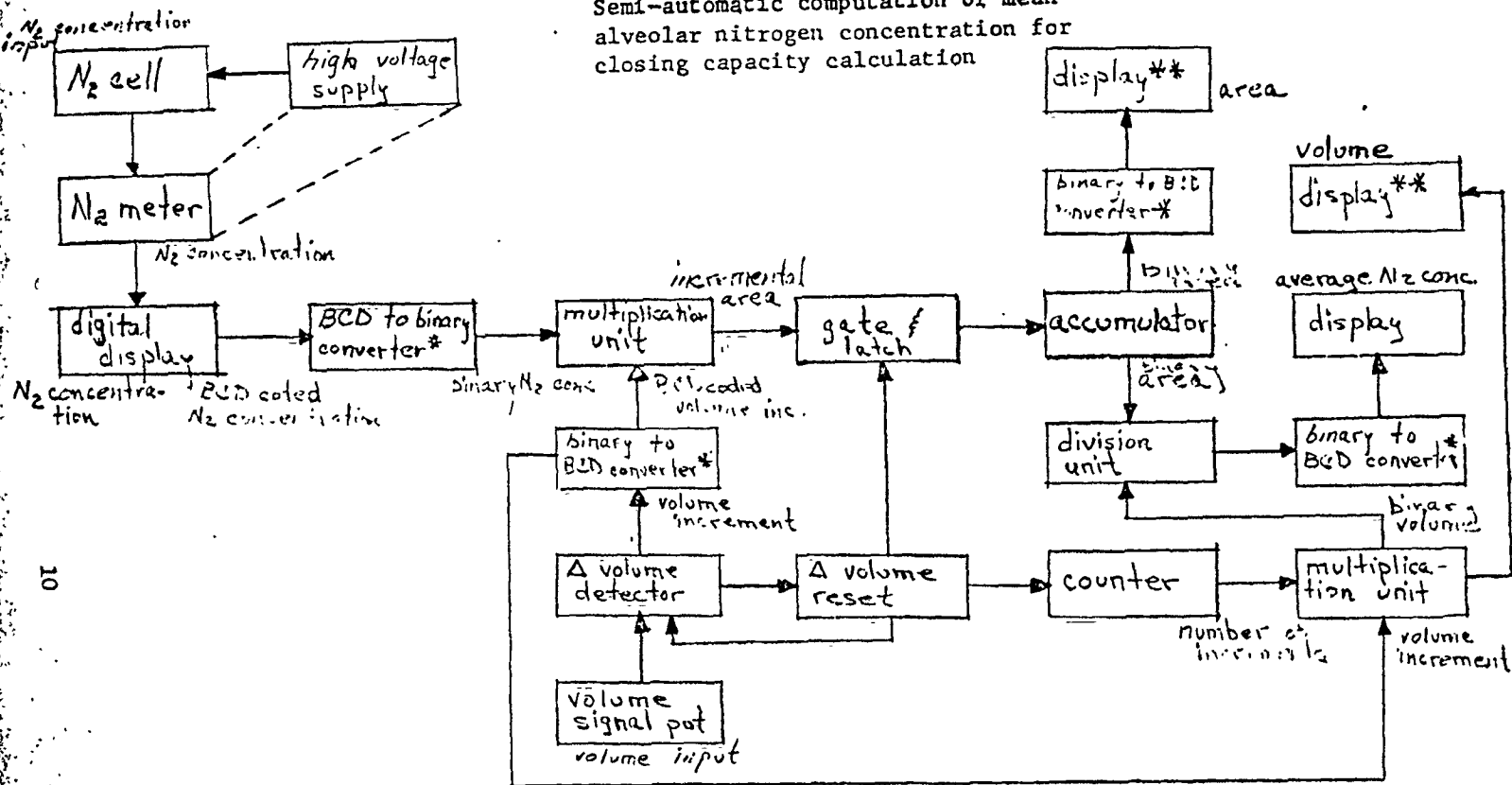
Responsible officer of institution

Typed Name David MintzerTitle Vice-President for Research & Dean of ScienceSignature David Mintzer Date 1/31/74Telephone R
Area Code Number Extension

1003540018

APPENDIX

Semi-automatic computation of mean alveolar nitrogen concentration for closing capacity calculation



* This flow diagram shows positions of all possibly needed binary to BCD and BCD to binary converters. The operating modes of the arithmetic units, BCD or binary addition, multiplication or division will determine which of the converters will be needed.

**** Area and volume displays are optional.**

1003540019

962

NORTHWESTERN UNIVERSITY
THE MEDICAL SCHOOL
303 E. CHICAGO AVENUE
CHICAGO, ILLINOIS 60611

DEPARTMENT OF COMMUNITY HEALTH
AND PREVENTIVE MEDICINE
312-649-7914

JEREMIAH STAMLER, M.D.
PROFESSOR AND CHAIRMAN AND
HARRY W. DINGMAN PROFESSOR OF CARDIOLOGY

January 28, 1974

Dr. Robert C. Hockett
Research Director
The Council for Tobacco Research
- USA, Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

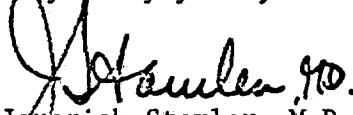
I'm heartily in accord with the objectives of the project being submitted by David W. Cugell, M.D. Dr. Cugell has been in charge of the pulmonary section of the Department of Medicine and director of the pulmonary function laboratories at this Medical Center for 18 years. He has recently shifted his interest from the intensive study of patients with established disease to the evaluation of asymptomatic patients using newly developed tests potentially capable of identifying patients in a very early, pre-symptomatic phase of bronchitis and emphysema.

As noted in the application I am the principal investigator for the Chicago portion of a nation-wide study of how to best intervene in male patients with a high risk of developing a myocardial infarct. Approximately 900 high risk men will be followed for a period of six years. Methods for identifying this high risk group, and procedures for follow-up and periodic testing are well established and have already been used in several other large scale epidemiological studies.

I welcome the opportunity of involving the patients to be enrolled in this intervention trial for an additional worthy project. We are now in the process of having this project approved as an ancillary study. With both coronary artery disease and chronic obstructive lung disease occurring predominantly in middle aged males, these coronary-prone men are an ideal group for evaluating the significance of newer tests of small airway function.

The combination of Dr. Cugell's knowledge and experience in lung function measurement and our extensive organization for collecting clinical and laboratory data provide an excellent opportunity to secure meaningful results.

Very truly yours,


Jeremiah Stamler, M.D.
Professor and Chairman

JS/ms

#547D - QUARANT

1003540021

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 12, 1974

Grant application No. 547D

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Jacobson, Liebow and Wyatt

Subject: Joseph J. Guarneri, Ph.D., L.I. Jewish-Hillside Medical Ctr., New Hyde Park
Continuation application No. 547D

"The influence of acute and extended exposure to cigarette smoke on the immunological function and metabolic activity of alveolar macrophages as related to pulmonary defense against inhaled bacteria".

History

CTR has supported this applicant since 1960. The current grant, for the last year of a three-year proposal, is in the amount of \$22,944 and expires June 30, 1974.

Therefore, the enclosed request competes without commitment.

Request

Application No. 547D requests \$51,835 plus two additional years.

Document Submitted

Enclosed is application dated 1/14/74, plus IV figures and XXVI tables.

Comment

The applicant states that his proposal summarizes 13 years of research supported by CTR. It is difficult to see what progress has been made recently, but staff will attempt to do so.

Another concern is lack of publication; nothing but abstracts have appeared since 1968. Staff will attempt to pursue this question with the applicant.

FWN:gh
Encls.

FWN
F.W.N.

1003540022

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

JAN 30 1974

Date: 1/24/74

1. Principal Investigator (give title and degrees):

Joseph J. Guarneri, Ph.D., Attending Microbiologist, Director Microbiology Labs.,
Long Island Jewish - Hillside Medical Center/Queens Hospital Center Affiliation.

2. Institution & address:

Long Island Jewish - Hillside
Medical Center
New Hyde Park, N.Y. 11040

Research Performance Site & Address
Long Island Jewish-Hillside Medical
Center/Queens Hospital Center Affiliation
82-68 164th Street, Jamaica, N.Y. 11432

3. Department(s) where research will be done or collaboration provided:

Division of Microbiology, Department of Laboratories.

4. Short title of study: The influence of acute and extended exposure to cigarette smoke on the immunological function and metabolic activity of alveolar macrophages as related to pulmonary defense against inhaled bacteria.

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

The proposed project is a direct investigation of the influence of acute and extended exposure to puffed cigarette smoke on the immediate immunological function of

7. Brief description of specific research aims:

alveolar macrophages in pulmonary defense; namely, the phagocytosis and destruction of inhaled bacteria. Methods are used that permit an evaluation of the effect of smoke-inhalation on the antibacterial capacity of alveolar macrophages under in vivo conditions of smoke-exposure and bacterial challenge. By means of paired and matched studies that utilize in vivo and in vitro quantitative methods of studying host defense mechanisms, it will be possible to study several immunological and biochemical parameters of alveolar macrophage function associated with antibacterial activity. Experiments are planned to permit the following evaluation of the effect of whole smoke and filtered smoke on the alveolar macrophage system and the reversibility of any adverse effects that may occur as a result of smoke inhalation: (a) phagocytosis and intracellular killing activity of alveolar macrophages under in vivo and in vitro conditions of bacterial challenge, (b) alveolar macrophage motility and cell adhesiveness as related to alveolar macrophage mobilization in response to inhaled bacteria and smoke, (c) the antibacterial activity and phagocytosis promoting properties of alveolar lining material and pulmonary secretions, (d) secretory IgA levels present in the lungs of animals under basal conditions and after exposure to bacterial aerosols, (e) oxygen uptake, glucose metabolism, lipid composition and fatty acid composition of alveolar macrophages harvested from animals under basal conditions and after bacterial provocation and (f) ATPase, hydrolytic enzyme, catalase, peroxidase activities and hydrogen peroxide production before and after bacterial challenge. This fundamental approach supports the project's specific aim to assess the influence of smoke-exposure on the alveolar macrophage system and to provide a rational basis for explaining the smoke data obtained in this laboratory: namely, cigarette smoke caused an impairment of the antibacterial activity that was totally reversible, and not apparent in macrophages harvested from smoke-exposed animals and then challenged with bacteria in the absence of cigarette smoke.

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8. Brief statement of working hypothesis:

2.

Background Material - Supporting Data - Working Hypothesis of Grant Proposal.

See pages 2A-1 to 2A-20

9. Details of experimental design and procedures (append extra pages as necessary)

See pages 2A-21 to 2A-33

1003540024

8. Statement of Working Hypothesis and Supporting Data: (Cited references on pages 2A-17 to 2A-20)

There is a great deal of information which documents the capacity of the normal lower respiratory tract to clear bacteria and remain sterile. This is indeed remarkable since bacterial contamination by inhalation and aspiration are a common occurrence. Evidence for the antibacterial activity of bronchopulmonary tissue of human beings and experimental animals, respectively, resides in the observations that bronchial secretions of normal subjects are sterile (1-3) and that bacteria deposited in the lungs of animals are rendered nonviable within hours (4-6). However, other studies also show that the bronchial secretions of chronic bronchitic patients contain respiratory pathogens (3) and that various experimental conditions, of suggested clinical importance, impede the pulmonary defense of mice against inhaled bacteria (4, 6-9).

Quantitative studies of pulmonary resistance to infection have been made possible by the development of techniques for simulating the natural mode of respiratory infection, namely, the inhalation of droplet nuclei laden with bacteria. This laboratory has developed, standardized and used for the past 10 years an aerosol system which permits the production of bacterial aerosols of controlled concentration and particle size from which predictable numbers of bacteria can be implanted in the lungs and trachea of experimental animals. By this method rapid lung clearance of a coagulase positive strain of Staphylococcus aureus by the murine lung has been demonstrated. One hour after aerosol exposure, 45% of the bacteria deposited are cleared: after 2 hours, 70%; 3 hours 80%; 4 hours 88%, and after 6 hours, 95% of the staphylococci are cleared. This clearance curve is remarkably constant for staphylococcal depositions between 10,000 and 300,000. Tracheobronchial clearance of the same strain of S. aureus is also effective and very rapid. In one hour 72% of the bacteria deposited on tracheobronchial tissue were cleared; at 2 hours, 85% and at 4 hours, 95% of the bacteria were cleared. Similar lung clearance rates for the removal of the same strain of S. aureus has been reported by other investigators (6, 7). Published data from our laboratory indicates that hypoxia, barbiturates, cigarette smoke and alcohol introduced immediately after bacterial aerosol exposure cause a reduction in lung clearance (4, 8, 9). Other published reports from our laboratory demonstrate that pretreatment with cortisone or endotoxin interferes with normal clearance (4, 8, 9) and the intestinal flora of the mouse exerts a profound influence on pulmonary resistance to an airborne bacterial challenge. Reports by other investigators demonstrate that acute starvation (7), cold (6), bacterial species (6), pre-exposure to a viral infection (11), ozone (12), silicosis (13), renal failure (14) and acidosis (15) markedly affect the normal process of removal of bacterial deposits by pulmonary tissue. It is of interest that abnormalities in normal lung clearance correlate better with indices of physiological dysfunction than with observable anatomical derangements. For example, experimental silicosis (13) caused minor reduction in lung clearance despite extensive anatomical derangement. In contrast, physiological circumstances such as acidosis (13) hypoxia (8), alcoholic intoxication (10) and barbiturates (8), cause inhibition of normal lung clearance without apparent damage to lung structure. Indeed the host factors which determine the outcome of the interaction between bacteria and lung tissue remain unclear. Reports from our laboratory clearly demonstrate the importance of genetic and environmental factors in pulmonary resistance to infection (8). We observed that two genetically related strains of mice differed solely in their capacity to withstand exposure to a single experimental condition, namely, cigarette smoke. It was also observed that the presence or absence of gram negative bacteria, specifically Escherichia coli predisposes mice to greater difficulty in handling inhaled bacteria, especially under the circumstance of smoke inhalation (8). In this regard, it has been demonstrated in our laboratory that circulating bacterial products such as E. coli endotoxin cause a marked interference with normal clearance of inhaled bacteria (8).

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The mucociliary system and alveolar macrophages comprise the major defenses of the respiratory tract. The individual effectiveness of each component has been demonstrated several times (15, 16) and newer techniques have made it possible to study in detail their individual participation in the disposal of foreign material from the lung (17-20). Attention has been focused on the importance of alveolar macrophages by studies which demonstrate that clearance of bacteria that are deposited at alveolar levels, is not primarily achieved by mechanical removal of bacteria by the mucociliary apparatus but is accomplished by the in situ bactericidal action of the lung (19). In addition, published reports from this laboratory (21-22) indicate that alveolar macrophages are mobilized in response to an airborne bacterial challenge. The inhalation of staphylococci provoked a 2.5 fold increase in macrophage numbers. Within 15 minutes after bacterial deposition, the numbers dropped 35%; but the elevated levels observed immediately after aerosol exposure were restored at the end of 30 minutes and then maintained with minor variation for a 4 hour period. The observation that whole-body x-irradiation (23) and immunosuppressive agents (24) suppress normal bacterial clearance, adds additional support to existing evidence that alveolar macrophages play an important role in pulmonary defense against inhaled bacteria. By utilizing recent refined techniques for securing alveolar macrophages in almost pure harvest, it has been possible to study the antibacterial and biochemical properties of alveolar macrophages. Under these circumstances published reports demonstrate that the alveolar macrophage system responds to a variety of experimental conditions that are epidemiologically associated with the genesis of pulmonary disease (8, 25). In this regard, published reports from this laboratory indicate that alcohol interferes with the mobilization of alveolar macrophages in response to a bacterial challenge but does not adversely affect cell viability (22). In contrast, cigarette smoke provokes an increase in the number of macrophages present in the lungs and does not adversely effect cell viability or interfere with macrophage mobilization by bacterial provocation (26). Cigarette smoke under in vivo condition of acute exposure to puffed cigarette smoke was observed to cause an inhibition of antibacterial activity that was reversible within hours (27) and the alveolar macrophage may act as the major detoxification mechanism available to the normal lung during acute exposure to cigarette smoke. Other investigators have shown that various inhaled noxious agents affect the function and number of alveolar macrophages. These agents include nitrogen dioxide (28, 29), ozone (30) and lead sesquioxide (31). In this regard, ozone, nitrogen dioxide and auto smog adversely affect the cell numbers (28,30), phagocytic ability (28,30) viability (32), hydrolytic enzymes (33) and interferon production (29) of the alveolar macrophage. Recent studies demonstrated that the alveolar macrophage is a specialized cell with metabolic and enzymatic characteristics distinct from most other white blood cells with phagocytic properties (34, 35). Its basal oxygen consumption is four times that of polymorphonuclear leukocytes, yet it requires only 20% increase during active phagocytosis. Peritoneal macrophages require a 250% increment for similar activities. In addition, the alveolar macrophage depends upon oxidative energy metabolism for its phagocytic function, and it has 3 to 4 times the lysozyme, acid phosphatase, and beta-glucuronidase activity of peritoneal macrophages (35). Recent studies (36) indicate that phagocytosis by alveolar macrophages is accompanied by the production of carbon dioxide ^{14}C from glucose -1 ^{14}C which is at least 5 times that observed with glucose -6 ^{14}C . The $^{14}\text{CO}_2$ production from both forms of labelled glucoses increases 4 times during active phagocytosis. The same laboratory (36) has presented data that suggests that alveolar macrophages exhibit peroxidative metabolisms and possesses 2 H_2O_2 utilizing pathways; namely, a nicotinamide adenine dinucleotide phosphate (NADP) linked cytoplasmic glutathione shuttle and a catalase dependent pathway. The significance of the H_2O_2 metabolism is uncertain. In the polymorphonuclear leukocyte, H_2O_2 constitutes an important component of an intracellular bactericidal system (36). Its role in the alveolar macrophage is less clear, since the alveolar macrophage lack white blood cell peroxidase, another important constituent of their bactericidal system. Several studies may be cited in which the metabolic

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function of alveolar macrophages are deranged by experimental agents that also interfere with bacterial clearance and the antibacterial activity of alveolar macrophages; cadmium (37) ions interfere with oxidative metabolism; and ozone depresses lysosomal hydrolase activity (38).

The role of the mucociliary apparatus as a major pulmonary defense mechanism has been recognized for years. It limits the number of inhaled particles that enter the lower respiratory tract and has the capacity to physically remove particles that are deposited at various levels of the lung endowed with ciliated cells. Inhaled bacteria that deposit on the ciliated surface of the trachea and bronchi are treated as particles and are subjected to the clearing action of the mucociliary apparatus. Certain environmental agents of clinical interest inhibit mucociliary function; alcohol (9), formic and acetic acid (39) and high concentrations of oxygen (40). Evidence for (41) and against (42) an adverse effect of smoke on particulate clearance of inhaled material may be cited. Viral infections (43), vitamin A deficiency (44) and chronic bronchitis (45) are specific diseases that affect the bronchial tract and also demonstrate an effect on the salient components of the mucociliary apparatus; namely, ciliary motion, mucus production and particle clearance. The recent observation, that bronchial mucus contains antibodies and several substances that exert non-specific bacteriostatic and bacteriocidal action against gram positive and gram negative bacteria (46) suggests that mucus or its components may contribute directly to bacterial killing of inhaled bacteria per se or provide the local conditions necessary for efficient alveolar macrophage activity. To correlate with this recent studies suggest that alveolar lining material (47) and local immune systems (SIgA) (48) may play a significant role in pulmonary defense against inhaled bacteria.

The experimental approach and studies proposed in the current grant request emanate from observations made in this laboratory regarding the influence of acute and extended exposure to cigarette smoke on pulmonary defense against inhaled bacteria under in vivo conditions of smoke-exposure and bacterial challenge. For this reason, a detailed review of the data accrued to date is indicated.

Acute exposure to cigarette smoke altered the normal pattern of tracheal and lung clearance of bacteria. (see 8A supporting data, results section I(A) - I(C) pages 2A-10). The introduction of cigarette smoke immediately after exposure to staphylococcal aerosols resulted in a decrease in the number of viable bacteria present in the trachea and a concomitant increase in the numbers of bacteria retained in the lung. Under the acute conditions of these experiments, the effect of cigarette smoke on tracheal clearance was found to be reversible. Alveolar macrophage studies have provided the following pertinent information. Smoke inhalation provoked a dose related increase in basal macrophage numbers and did not adversely affect cell viability. In addition, exposure to cigarette smoke after staphylococcal aerosol challenge did not interfere with the mobilization of macrophages in response to a bacterial challenge. These findings demonstrate that one of the immediate effects of smoke inhalation on the cellular defense mechanisms of the respiratory tract is a direct stimulation of the alveolar defense system without an adverse effect on cell viability. To correlate with this, smoke-exposure did not provoke the migration or mobilization of other phagocytic cells such as polymorphonuclear leukocytes to pulmonary sites. The data further suggests that the presence of increased macrophages may play a central role in protecting the lung against smoke toxicity. To postulate that these cells play a protective role is supported by studies presented as supporting data in this report which demonstrate the following: (1) increased numbers of macrophages reduce or limit the adverse effect of cigarette smoke on the antibacterial activity of a macrophage population in vitro and (2) alveolar macrophages are capable of inactivating toxic components present in a smoke solution that impair the

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the antibacterial activity of normal alveolar macrophages in vitro.

The data obtained from a direct investigation of the phagocytic and bactericidal properties of alveolar macrophages under in vivo conditions of smoke-exposure has provided additional pertinent information. (see 8A Supporting Data, Results Section II(A) - II(C) pgs.2A-11 to 2A-12) As demonstrated by the pulmonary lavage studies, exposure to cigarette smoke caused an accumulation of "toxic substances" that inhibit the antibacterial activity of alveolar macrophages. However, smoke inhalation did not permanently impair the antibacterial activity of the alveolar defense system. Specifically, smoke-exposure caused an impairment of the phagocytic and bactericidal powers of macrophage harvests that was readily reversible within hours. The fact that macrophage function was restored under in vitro conditions of recovery; namely by multiple washing and incubation of macrophage in a smoke-free environment demonstrates the capacity of the alveolar macrophage system to cope with cigarette smoke and the importance of in vivo processes which effectively remove inhaled products of cigarette smoke that influence macrophage function either directly or indirectly by altering the immediate environment in which macrophages perform their phagocytic function. To correlate with this, the adverse effect of smoke inhalation was reversed under in vivo conditions of recovery, namely by housing smoke-exposed animals in a smoke-free room prior to obtaining lung harvests. Collectively, these observations suggest that the respiratory tract responds to the presence of cigarette smoke, as well as other foreign materials in a similar manner; namely, pulmonary defense mechanisms are activated and respond by removing and/or inactivating smoke products deposited in the lung. It appears reasonable to assume that the efficacy of this response is facilitated by the in vivo mobilization of increased macrophage numbers in relation to the inhalation of cigarette smoke. Such an interpretation of the data would be in keeping with experimental evidence previously cited in this report which demonstrates that alveolar macrophages may play a key role in protecting the lung against the toxic effects of cigarette smoke.

Studies performed under in vitro conditions of smoke-exposure have served to provide: (1) the laboratory techniques necessary for more meaningful studies of the effect of cigarette smoke on the living and functioning lung and (2) a better understanding of the conditions of bacterial challenge and smoke-exposure that influence the antibacterial properties of alveolar macrophages. As demonstrated by studies presented in this report (see 8A Supporting Data, Results Section III (A) - III (F) pgs.2A-12 to 2A-15) the antibacterial capacity of a macrophage population in the absence and presence of cigarette smoke was enhanced by the presence of increased numbers of alveolar macrophage. Specifically, the presence of increased numbers of macrophages reduced or limited the immediate adverse effect of cigarette smoke on alveolar macrophage function. In addition, macrophage cultures inactivated toxic components of a smoke solution that impair the antibacterial activity of alveolar macrophages in vitro. These findings support published reports which have focused attention on the importance of alveolar macrophage mobilization as a pulmonary defense mechanism against inhaled infectious and toxic agents. Results of other studies presented in this report indicate that the local conditions necessary for effective antibacterial activity by normal and smoke-exposed macrophages differ. In this regard, the antibacterial activity of macrophages during smoke-exposure was significantly influenced by the concentration of serum in the culture medium. This effect could not be accounted for by the presence or absence of an added carbon source (glucose) in the culture medium or the increased acidity of the culture medium induced by cigarette smoke. The fact that exposure to cigarette smoke interfered with the antibacterial activity of alveolar macrophages only at serum concentrations of 2.5% or less indicates that serum factors may protect against smoke toxicity. This may be related to specific immunological components of serum. In addition, it has also been observed that the protective action of serum is reduced if cigarette smoke is introduced prior to

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bacterial challenge. Specifically, prior exposure of macrophage cultures to cigarette smoke reduced the amount of cigarette smoke necessary to suppress antibacterial activity. This difference may be a reflection of the long term effects of cigarette smoke on a static macrophage population and probably relates to several factors including the effect of extended exposure to cigarette smoke on: (1) serum factors which promote and sustain effective macrophage function and (2) the physiological state of macrophages at the time of bacterial challenge. Macrophages obviously respond to the presence of cigarette smoke by removing and/or detoxifying smoke products in their environment. The fact that the adverse effects of in vitro smoke-exposure was partially reversed by multiple washing of macrophage cultures demonstrates the beneficial results derived from reducing the amount of smoke in contact with the alveolar cells and the importance of the cleansing action of increased numbers of macrophages (mobilization) and mucociliary activity in the live and functioning lung. Data has also been presented which clearly demonstrates that exposure to whole smoke drawn through a glass fiber filter reduced the adverse effect of in vitro smoke-exposure on the antibacterial activity of macrophages. Specifically, filtered whole smoke did not interfere with phagocytic activity but continued to impair bacterial killing. These observations suggest that the particulate and gas phases of whole smoke may have separate effects on macrophage function.

Marked differences in the effect of in vivo and in vitro smoke-exposure on macrophage function have also been noted. In vitro conditions of smoke-exposure produced an impairment of antibacterial activity that was not totally reversed by multiple washing and incubation of macrophages in smoke-free tissue culture flasks. The differences in the effect of in vivo and in vitro smoke-exposure on macrophage function may be attributable to several factors including: (1) differences in the amount of smoke that macrophages were exposed to under both experimental conditions and (2) the static and unphysiological conditions that prevail under in vitro conditions of smoke-exposure. The latter interpretation of the data would be in keeping with the mobilization and detoxification data reported herein.

Pulmonary studies of the influence of extended exposure to puffed cigarette smoke on pulmonary defense against inhaled bacteria under in vivo conditions of smoke-exposure and bacterial challenge have also been completed (see 8A Supporting Data, Results Section IV(A)-IV(D) pgs. 2A-15 to 2A-16). Exposure to cigarette smoke for one hr daily for 3, 5, 10 and 15 days prior to bacterial aerosol challenge altered pattern of normal clearance. Specifically, smoke inhalation reduced the initial clearance rate of inhaled bacteria but normal clearance rates were restored with minor variation within hours after the termination of smoke-exposure. Under the conditions of these experiments, smoke inhalation provoked a large increase in basal macrophage numbers and did not adversely affect cell viability. In addition, cigarette smoke did not mitigate the mobilization of alveolar macrophages in response to bacterial provocation or provoke the presence of polymorphonuclear leukocytes in lung harvests. The data obtained from studies of the phagocytic and bactericidal properties of alveolar macrophages under in vivo conditions of extended exposure to cigarette smoke has provided the following information. The antibacterial activity of alveolar macrophages harvested from smoke exposed animals washed several times prior to bacterial challenge was unimpaired. A full report of these observations, as well as others pertaining to the influence of extended exposure to cigarette smoke on pulmonary defense will be submitted at the end of the current year of grant support.

The proposed grant request is a direct extension of the work completed in this laboratory concerning the influence of acute and extended exposure to cigarette smoke on pulmonary defense mechanisms. This project is designed on the basis of established evidence that alveolar macrophages play a dominant role in disposing of inhaled bacteria (18,19,21) and takes into consideration current information relating to the

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presence in the bronchopulmonary tree of secreted fluids, both immunological and and non-immunological in character (49) that contribute to pulmonary defense and may act as the mediators of effective alveolar macrophage function. In addition, the grant proposal takes into account several biochemical parameters of alveolar macrophage function that are associated with the effective antibacterial activity of phagocytes. To correlate with this, evidence may be cited which demonstrates an apparent contradiction; namely, the effective antibacterial powers of alveolar macrophages in the intact and functioning lung(4-9) and the diminished bactericidal capacity (50,51) under in vitro conditions of study. The experimental design also acknowledges that meaningful animal studies must be performed under in vivo conditions of smoke-exposure and utilizes a smoke generating and exposure chamber that produces puffed cigarette smoke of predictable and controlled concentration. In this regard, the results obtained in this laboratory under in vivo conditions of smoke-exposure (See Supporting Data, Results Section, pgs. 2A-10 to 2A-16) demonstrated that smoke-exposure produced an impairment of the antibacterial activity of alveolar macrophages that was readily reversible and not apparent in the absence of cigarette smoke or smoke products. These findings are in good agreement with the data obtained from studies (52) with alveolar macrophages harvested from human smokers and are at variance with the results (53) obtained with rabbit alveolar macrophages exposed to cigarette smoke under in vitro conditions of smoke-exposure.

Experiments are detailed which permit a direct investigation of the influence of acute and extended exposure to cigarette smoke on the immediate immunological functions of alveolar macrophages in pulmonary defense; namely, the phagocytosis and intracellular destruction of inhaled bacteria. By utilizing in vivo and in vitro quantitative methods of studying phagocyte response to bacterial provocation, studies are planned to investigate several parameters of alveolar macrophage function, associate with its central role in pulmonary defense against inhaled bacteria in the normal situation and during the inhalation of cigarette smoke. To this end, studies are planned to investigate the effect of smoke inhalation on the phagocytic and intracellular killing activity under in vivo and in vitro conditions of bacterial challenge. Separate studies are detailed to assess the influence of cigarette smoke on macrophage mobility and adhesiveness because they represent critical aspects in the in vivo mobilization of macrophages in response to an airborne bacterial challenge and cigarette smoke. Additional studies are proposed to evaluate the influence of alveolar lining material, Secretory IgA and pulmonary secretions on antibacterial activity of alveolar macrophages harvested from control and smoke-exposed animals. The information is needed to delineate the relative role of alveolar macrophages and secreted pulmonary fluids in pulmonary defense and to assess the relative susceptibility of each component to smoke inhalation. In addition, metabolic studies will be performed to assess the effect of cigarette smoke on ATPase activity, respiration, glucose metabolism, lactic acid production, catalase activity, peroxidase activity, hydrogen peroxide production, lipid composition, fatty acid composition, hydrolytic enzyme activity of alveolar macrophages which underlie or are concomitant with normal antibacterial activity. This information is desirable, since respiration, glucose metabolism, ATPase activity and lipid metabolism represent important links in the successful mobilization and subsequent antibacterial activity of alveolar macrophages. Indeed impaired lipid metabolism may compromise membrane phenomena associated with phagocytosis and derangements of peroxidative metabolism and/or the intracellular distribution and activity of hydrolytic enzymes may profoundly affect the bactericidal capacity of lung phagocytes. This fundamental approach supports the project's specific design to investigate: (a) the influence of acute and extended exposure to cigarette smoke on the immediate immunological function of alveolar macrophages in pulmonary defense against inhaled bacteria and (b) provide a better understanding of the relative role of alveolar macrophages in the cellular response of the respiratory tract, in the normal situation and during the inhalation of cigarette smoke.

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8 (A) Supporting DataMaterials and Methods

I. Conditions of Bacterial Clearance Studies: Since bacterial clearance studies comprise a major part of the proposed research project, a review of the method of aerosol formation, exposure and quantitative aspects of bacterial clearance is indicated. Aerosols are generated from a buffered suspension (pH 7.3) of staphylococci contained in 8 glass nebulizers. The initial spray from the nebulizers is directed into mixing chambers through which a secondary airflow of 100 cubic feet/min. is drawn. The large volume of secondary air serves to mix, dilute and dry the initial bacterial aerosol; direct it past an interposed baffle for removal of large droplets; and then carry it through a large plexiglass exposure chamber that can accommodate up to 200 mice. The particle size distribution of the bacterial aerosol is monitored with an Andersen Sampler. White male Swiss Webster mice are divided into groups of 8 to 10 animals and exposed to Staphylococcal aerosols for 30 min. Immediately after exposure (0 time), one group of animals is sacrificed to determine the numbers of viable bacteria deposited in the trachea and lungs, respectively. The trachea and lungs are removed as separate blocks and individually ground in glass homogenizers. The remaining groups of challenged mice are killed and processed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after aerosol exposure. Bacterial counts are obtained from nutrient agar pour plates of lung and tracheal tissue homogenates. By this method paired studies of lung and tracheal clearance of bacterial deposits are possible. By subtracting the mean number of culturable bacteria retained in the lung at each interval during the post aerosol exposure period from the number originally deposited (0 time) the mean number of staphylococci cleared by the lung may be derived. From these data lung clearance rates are calculated by expressing the number retained in the lung as a percentage of the deposition number (0 time) and subtracting this value from 100%. Similarly, from the numbers of bacteria present in the trachea at 0 time and at various intervals after aerosol exposure, the number and percent bacteria cleared by the trachea may be obtained.

II. Conditions of In Vivo Smoke-Exposure: It is apparent that meaningful studies of the influence of cigarette smoke on pulmonary resistance against inhaled bacteria require strict experimental circumstances. The concentration and composition of the smoke must be controlled and clearly defined. Variations in smoke generation are invariably accompanied by changes in the amount of tar and may influence the concentration and/or composition of the volatile components of the gas phase. In addition, studies under in vivo and in vitro conditions of smoke exposure demonstrate the importance of a dose-response relationship in the interpretation of the biological effects of cigarette smoke on the major defense mechanisms of the lung; namely, mucociliary function and alveolar macrophage activity.

The smoke exposure system is schematically presented in Figure I. Puffed cigarette smoke generated from non-filtered, 70 mm cigarettes is drawn into a stainless steel mixing chamber and through a smoke exposure chamber by a secondary airflow of room air. Animals in the control chamber are exposed to puffed air drawn through an unlighted cigarette directed through a stainless steel mixing chamber by a secondary air flow. The smoke exposure and control chambers are made of transparent plexiglass which permit observations of animals during experiments. Trap doors located on the top of each chamber facilitate the introduction and removal of a 4-tiered stainless steel rack that accommodates 40 mice housed in individual rubber mesh containers. The downstream ends of the smoke exposure and control chambers are attached to separate stainless steel evacuation chambers that are connected by a common cylinder to the negative pressure side of an air blower. Sampling ports located in the evacuation chambers permit the introduction of sampling devices into the exposure chambers to obtain data relative to the particulate and gas phases of cigarette smoke, temperature and

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relative humidity. The effluent from both the smoke exposure and control chambers is ultimately eliminated through a pipe inserted into an air duct that is vented outside the building. As shown in Figures 1A and 1B, a continuous stream of puffed cigarette smoke is produced by an automatic smoke machine* designed to sequentially puff 30 cigarettes and adjusted to deliver a 35 ml puff of 2 seconds duration from each cigarette once every minute. The smoke generating apparatus consists of an index disc and wheel assembly programmed to position and maintain each lighted cigarette for 2 seconds at the intake port of the smoke aspirator. An airflow of 1.05 liters per minute through the intake port was established by introducing compressed air at a rate of 14.0 liters per minute into the air inlet of the smoke aspirator. Air flow into the air inlet of the smoke aspirator is measured with a calibrated rotometer and controlled by a needle point valve positioned between the air compressor and rotometer. The smoke exhaust of the aspirator is placed inside the open end of a stainless steel mixing chamber in which the cigarette smoke is further diluted and directed across the smoke exposure chamber by a flow of room air through the apparatus. This secondary air flow is established by the air blower at the downstream end of this apparatus. A rotometer and needle point valve are used to control the volume and rate of air flow.

The aspirator was calibrated by taking simultaneous measurements of the air flow at the air inlet and smoke exhaust orifices with a rotometer, pneumotachograph, electronic amplifiers and recorders. From these data, the gas volume drawn through a cigarette positioned at the smoke intake port was derived. The relationship between the volume of air introduced into the air inlet and drawn through a lighted cigarette is plotted in Figure II.

Separate studies were undertaken to obtain accurate determination of the volume and flow rate of room air through the apparatus. For this purpose an orifice meter and manometer were introduced into the evacuation chambers of the control and smoke chambers as indicated in Figure III. Simultaneous measurements taken with a manometer and rotometer provided the data plotted in Figure IV. These results indicate that a secondary air flow of 30 to 100 liters per minute may be maintained in the smoke exposure chamber.

The data obtained from the above experiments has provided the information and techniques necessary to: (a) define the conditions of smoke exposure during studies of the influence of cigarette smoke on pulmonary resistance to infection. (b) standardize the conditions of smoke exposure from study to study. (c) assess the effect of different concentrations of cigarette smoke on alveolar macrophage and mucociliary function, (d) plan and implement a protocol to monitor the particulate and gas phases of cigarette smoke during animal studies and (e) assess the relative importance of the particulate and gas phases of cigarette smoke on host resistance to pulmonary infection.

III. Conditions of In Vitro Smoke-Exposure: A commercial brand of non-filtered cigarette was attached to a 30 ml. syringe by a rubber tube and smoke was produced by withdrawing the barrel of the syringe at a rate of approximately 18 ml. per second. Six successive "puffs" of cigarette smoke were introduced into the syringe and emptied by removing a rubber tube containing the lighted cigarette. The smoke from the seventh puff was introduced into the tissue culture flask with a sterile hypodermic needle. The protocol of each study included control flasks containing only bacteria and alveolar macrophages challenged with bacteria respectively. Under these conditions, both categories of tissue culture preparations were exposed for 1.5 hours to whole cigarette smoke or whole smoke drawn through a glass fiber filter**.

*Progressive Engineering Co., Richmond, Va.

** Cambridge glass fiber filter, Phipps and Bud, Richmond, Va.

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IV. In Vitro Phagocytosis System: Alveolar macrophages were harvested from the lungs of rabbits weighing 1.0 to 2.0 kg by pulmonary lavage. The animals were sacrificed by injecting air into the external marginal vein. The trachea and lungs were exposed and macrophages harvested by washing out the intact lungs two times with 17 ml of the suspending medium used in the in vitro phagocytosis system (described below). The harvested cellular lung contents were recovered by centrifugation at 2000 rpm for 20 min. Total cell counts were performed in a bright line hemacytometer, and differential counts made on Wright stain smears.

The harvested alveolar macrophages were suspended in Hank's solution, diluted to a final cell count of 2.0 to 4.0×10^6 cells and transferred into 30 ml plastic tissue culture flasks. The total fluid volume in each flask was 3.0 ml containing 2.0 to 4.0×10^6 macrophages, 2.0 to 4.0×10^6 Staphylococcus aureus and normal rabbit serum at various concentrations in accordance with the protocol of each study. The pH of control and macrophage containing flasks was determined with a Beckman zeromatic pH meter immediately after inoculation and at the end of each incubation period.

Quantitative studies of the phagocytic and intracellular killing activity of alveolar macrophages require the enumeration of the total number of viable bacteria present in the extracellular fraction and macrophage fraction of each preparation. In addition, the stability of the bacteria in the suspending medium as well as multiplication of extracellular bacteria must be monitored. For this purpose, control flasks devoid of macrophages but containing suspending medium were included in the protocol of each experiment. In every study, several flasks containing macrophages and control flasks were inoculated with bacteria. Immediately after bacterial challenge (0 time) and at various intervals after incubation at 37°C , replicate flasks were removed from the incubator and processed. The extracellular fraction was isolated by removing the supernatant from the macrophage cultures with a pipette and washing the macrophage monolayer 3 times with 2.5 ml of suspending medium. To recover the macrophage fraction, cell monolayers were washed 3 times with 2.5 ml of sterile distilled water to lyse the macrophages adhering to the surface of the tissue culture flask. To insure complete separation of the cells from the flask, 1 ml of glass beads was added after the addition of distilled water and shaken on a Vortex mixer for 1 min. The completeness of cell separation was confirmed by microscopic examination of the tissue culture preparation. The contents of the control flasks were removed with a pipette and the flasks washed 3 times with suspending medium. The extracellular fraction, macrophage preparation and contents of control flasks were placed in an ice water bath and bacterial counts were obtained from nutrient agar pour plate incubated at 37°C for 24 hrs. Bacterial counts were taken from separate pairs of control and macrophage containing flasks immediately after introduction of S. aureus (0 hr.) and at various intervals after incubation at 37°C . From the number of viable S. aureus present in the control flask (A) extracellular fraction (B) and macrophage fraction (C) at each interval after bacterial challenge, the % bacteria phagocytized, % phagocytized bacteria surviving within macrophages and % bacteria cleared by macrophages were determined as follows:

$$(1) \text{ \% phagocytized} = \frac{A - B}{A} \times 100 \quad (2) \text{ \% intracellular survival} = \frac{C}{A - B} \times 100$$

$$(3) \text{ \% killed} = \frac{B + C \text{ at each interval after bacterial challenge}}{B + C \text{ at 0 hr.}} \times 100 - 100\%$$

Separate studies were performed in which inactivation of intracellular bacteria was assessed independent of phagocytosis. For this purpose several macrophage cultures were challenged with S. aureus as described above for 30 min. At this time, the supernatant was discarded and the surface of each macrophage cultures washed with Hank's solution to remove all extracellular bacteria and thus terminate the phagocytosis of additional bacteria. Bacterial counts were obtained in triplicate immediately after termination of phagocytosis and at various hourly intervals.

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ResultsI. The Influence of Acute Exposure to Puffed Cigarette Smoke on Pulmonary Clearance of Inhaled Staphylococcus Aureus Under In Vivo Conditions of Bacterial Challenge and Smoke-Exposure.

(A) The Effect of Acute Exposure to Cigarette Smoke on Tracheal and Lung Clearance: Large numbers of mice were exposed to aerosols of *S. aureus* for 30 minutes. Immediately after aerosol exposure one group of mice was sacrificed to determine the number of bacteria deposited in the trachea and lungs, respectively. The remainder of the bacteria-challenged mice were divided into smoke and control groups and exposed to cigarette smoke and a secondary air flow of room air, respectively. As shown in Table I, within 15 minutes after exposure to cigarette smoke, there was a 2.5 fold decrease in the numbers of staphylococci culturable from the trachea, and this decrease in viable bacteria was observed with minor variation throughout the remainder of the 4 hour post aerosol exposure period. This effect was accompanied by an increase in the numbers of bacteria retained in the lung. At the end of 30 minutes, the smoke exposed mice retained 1.4 times as many bacteria as controls. Bacterial retention was further increased 2.1 to 2.5 times control numbers by the inhalation of cigarette smoke for 1 and 4 hours, respectively. As shown in Table II the effect of cigarette smoke on tracheal clearance was reversible. Within 3 hours after the termination of smoke exposure, bacterial counts obtained from the trachea of control and smoke exposed mice were not significantly different.

Conclusions: Cigarette smoke altered the normal pattern of tracheal and lung clearance. Smoke inhalation resulted in a decrease in the number of bacteria present in the trachea and a concomitant increase in the number of viable bacteria retained in the lungs. The effect of cigarette smoke on tracheal clearance was reversible.

(B) The Effect of Cigarette Smoke on Basal Macrophage Yields: The effect of cigarette smoke on the numbers of alveolar macrophages harvested from the lungs of mice was studied by comparing the macrophage yields obtained from untreated mice (Basal Group), mice exposed to a secondary air flow (Control Group) and animals challenged with puffed cigarette smoke diluted by a secondary air flow. The data presented in Table III demonstrates that cigarette smoke provokes a reproducible increase in macrophage numbers. The latter effect was found to be dose related. In addition, exposure to cigarette smoke for 4 hours did not adversely affect macrophage viability (Table IV).

Conclusions: Smoke inhalation provokes a dose related increase in basal macrophage numbers and does not adversely affect cell viability.

(C) The Effect of Cigarette Smoke on the Mobilization of Alveolar Macrophages in Response to a Bacterial Challenge: For this purpose basal macrophage yields were obtained from untreated mice and the remainder were exposed to an aerosol of *S. aureus* for 30 minutes. Immediately after aerosol exposure one group of mice was sacrificed to determine the numbers of bacteria deposited in the lung and the remainder of the challenged mice were divided into Control and Smoke groups. As indicated in Tables V and VI, the macrophage yields obtained from Control and Smoke exposed mice were the same over an entire 4 hour post aerosol exposure period.

Conclusions: Smoke inhalation did not interfere with the mobilization of alveolar macrophages in response to a bacterial challenge.

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II. The Influence of Acute Exposure to Puffed Cigarette Smoke on the Antibacterial* Activity of Alveolar Macrophages: In Vivo Smoke-Exposure and In Vitro Bacterial Challenge:

(A) The Antibacterial Activity of Alveolar Macrophages Incubated in the Pulmonary Lavage Solution Obtained from the Lungs of Control and Smoke-Exposed Rabbits: For this purpose, the lungs of smoke-exposed and control rabbits were washed with Hank's solution containing 0.1% glucose and 2.5% normal rabbit serum. The recovered lung lavage was then separated by centrifugation into an acellular fraction (pulmonary lavage solution) and a cellular fraction containing macrophages. In each study, the antibacterial activity of control macrophages was evaluated after incubation for 1.5 hours in pulmonary lavage solution obtained from smoke-exposed and control animals.

As shown in Table VII, incubation of control macrophages in control pulmonary lavage solution resulted in effective antibacterial activity. At the end of 1.5 hours, the macrophage cultures phagocytized 55% and killed 40% of the staphylococci. In contrast, control macrophages incubated in pulmonary lavage solution obtained from smoke-exposed animals showed a 27% decrease in the percent bacteria phagocytized and a 25% decrease in the percent bacteria killed.

Conclusions: The impairment of the antibacterial activity of alveolar macrophages by cigarette smoke inhalation may be directly related to the presence or accumulation of "smoke products" in the lung. "Smoke products" may exert their adverse effect on the alveolar macrophage defense system directly by compromising the antibacterial capacity of existing macrophages or indirectly by altering the immediate environment in which macrophages perform their phagocytic function.

(B) The Effect of Cigarette Smoke on the Antibacterial Activity of Alveolar Macrophages: In Vivo Smoke-Exposure and In Vitro Bacterial Challenge. In order to compare the antibacterial activity of smoke-exposure and control rabbits, experimental and control animals were exposed to puffed cigarette smoke and the secondary air flow, respectively, for 1.5 hours. At this time, alveolar macrophages harvested on both categories of animals were placed in tissue culture flasks and immediately challenged with *S. aureus*. Other studies were performed to determine if changes in the antibacterial activity of macrophages harvested from smoke-exposed animals were reversible. The reversibility studies were performed under two different experimental recovery conditions. In one series of studies, macrophage harvests from smoke-exposed animals were incubated in tissue culture flasks for 1.5 hours (in vitro recovery) prior to bacterial challenge. In the second study series, in vivo circumstances of recovery were established. Rabbits were first exposed to cigarette smoke for 1.5 hours and then placed in a smoke-free room for another 1.5 hours. At this time, the macrophages were harvested and challenged with staphylococci. Appropriate controls were included in every study. As shown in Table VIII, the antibacterial activity of alveolar macrophages harvested from animals exposed to whole cigarette smoke for 1.0 and 1.5 hours, respectively, was impaired. The data presented in Table IX clearly demonstrates that the adverse effect of cigarette smoke on the antibacterial activity of alveolar macrophage was reversed by incubation in a smoke-free tissue culture flask for 1.5 hours prior to bacterial challenge (in vitro conditions of recovery). Similarly, as shown in Table X, the smoke effect was reversible under in vivo conditions of recovery; specifically, the alveolar macrophage harvested from smoke-exposed animals housed in a smoke-free room for 1.5 hours prior to macrophage harvest phagocytized and destroyed staphylococci as effectively as macrophages from control animals.

Conclusions: Exposure of alveolar macrophages to whole cigarette smoke under in vivo conditions of smoke-exposure caused an impairment of the antibacterial activity of alveolar macrophages that was reversible within hours.

* % changes in antibacterial activity of smoke-exposed macrophages derived as follows:
% phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

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(C) The Effect of Multiple Washings of Alveolar Macrophages Harvested from Smoke-Exposed Animals on Cigarette Smoke Induced Changes in Antibacterial Activity: Studies of the reversibility of the adverse effect of cigarette smoke on macrophage function were performed under in vitro condition of recovery. Macrophages harvested from smoke-exposed and control animals were washed three times with Hanks' solution and then challenged with *S. aureus*. In addition, each study also included macrophage harvests from both categories of animals that were not washed prior to bacterial challenge. As shown in Table XI, compared to unwashed controls, the percent bacteria phagocytized by unwashed macrophages harvested from smoke-exposed animals was decreased 22%, the percent intracellular survival of phagocytized bacteria was increased 26%, and the percent bacteria killed decreased 25%. In contrast, the alveolar macrophages from smoke-exposed animals washed prior to bacterial challenge showed the same phagocytic and bactericidal powers as macrophages harvested from control animals.

Conclusions: Multiple washing of alveolar macrophage harvested from smoke-exposed animals completely reversed the adverse effect of smoke inhalation on macrophage function.

III. The Effect of Cigarette Smoke on the Antibacterial Activity* of Alveolar Macrophages: In Vitro Smoke-Exposure and In Vitro Bacterial Challenge: For this purpose alveolar macrophages harvested from untreated rabbits were placed in tissue culture flasks containing Hanks' solution supplemented with 0.1% glucose and 2.5% normal rabbit serum. In one series of studies, 8 ml. of cigarette smoke from a commercial brand of non-filtered cigarette, were introduced and maintained for 1.5 hours immediately after bacterial challenge. Under these conditions, studies were performed to determine the influence of alveolar macrophage numbers on the bactericidal capacity of an in vitro antimicrobial system during smoke-exposure. In a second series of studies, macrophage cultures were first exposed to cigarette smoke for 1.5 hours, and then challenged with bacteria. Cigarette smoke was introduced as an experimental condition by subjecting macrophages to either 8 ml. of whole smoke or Hanks' solution exposed to 2 ml. of whole smoke for 1.5 hours prior to use as a culture medium. Under these circumstances of smoke-exposure, studies were performed to determine if changes in the antibacterial activity of smoke-exposed macrophages were reversible. In addition, "detoxification" studies were carried out to determine the ability of alveolar macrophages to eliminate constituents of smoked Hanks' solution that depress antibacterial activity.

(A) The Influence of Macrophage Numbers on the In Vitro Antibacterial Activity of Alveolar Macrophages During Smoke-Exposure: Studies were performed in which macrophage-bacterium ratios (M/B) of 2:1, 3:1 and 4:1 were established by challenging 4×10^6 , 6×10^6 , 8×10^6 macrophages, respectively, with 2×10^6 staphylococci.

The data presented in Table XII indicate the following: (1) at each macrophage-bacterium (M/B) ratio studied, exposure to 8 ml. of cigarette smoke interfered with the phagocytosis and intracellular destruction of bacteria and (2) as the ratio of macrophages to bacterium (M/B) is increased, there is a significant decrease in the adverse effect of 8 ml. of cigarette smoke on the antimicrobial properties of alveolar macrophages. As shown in Table XII, at a M/B ratio of 2:1, the percent bacteria killed by smoke-exposed macrophages was decreased 27% compared to corresponding controls. In contrast, at M/B ratios of 3:1 and 4:1, the percent staphylococci killed by smoke-exposed macrophages was decreased 20% and 12%, respectively.

Conclusions: The presence of increased numbers of macrophages reduces or limits the adverse effect of cigarette smoke on alveolar macrophage function in an in vitro antimicrobial system.

* % changes in antibacterial activity of smoke-exposed macrophages derived as follows:
% phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

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(B) The Action of Alveolar Macrophages on the "Toxic" Components of Smoked Hanks' Solution: A smoke solution was prepared by introducing 2 ml. of whole smoke into 30 ml. tissue culture flasks containing 3 ml. of Hanks' solution supplemented with 0.1% glucose and 2.5% normal rabbit serum. Alveolar macrophage harvests obtained from several rabbits were pooled and used in experiments specifically designed to provide the following information: (1) the effect of a smoke solution on the antibacterial activity of alveolar macrophages, (2) the reversibility of changes in macrophage function induced by the smoke solution and (3) the effect of alveolar macrophages on the constituents of a smoke solution that may influence antibacterial activity. This was accomplished by assessing the antibacterial activity of alveolar macrophages under the following conditions of bacterial challenge: (A) Experimental Conditions I - In order to investigate the immediate toxic effect of the smoke solution on antibacterial activity, macrophage cultures were exposed to smoked Hanks' solution and *S. aureus* introduced simultaneously and maintained for 1.5 hours, (B) Experimental Conditions II - Reversibility studies were performed in which macrophage cultures were first exposed to smoked Hanks' solution for 1.5 hours. At this time, the smoke solution was removed by pipette and set aside for future use. The macrophage cultures were then provided with fresh unsmoked Hanks' solution and challenged with staphylococci for 1.5 hours and (C) Experimental Condition III - To study the influence of alveolar macrophage activity on the components of a smoke solution that affect macrophage function, macrophage cultures were challenged with *S. aureus* during incubation in the smoke solution previously set aside after recovery from the tissue culture flasks described in Experimental Condition II. The bacteria and recovered smoke solution were introduced simultaneously and maintained for 1.5 hours.

The data presented in Table XIII indicates that smoked Hanks' solution caused an irreversible impairment of the antibacterial activity of alveolar macrophages. As shown in Table XIII (Experimental Condition I) compared to controls, macrophage cultures exposed to smoked Hanks' solution and *S. aureus* simultaneously, showed a 16% decrease in the percent bacteria phagocytized and an 18% decrease in the percent bacteria killed. Comparable results were obtained with macrophage preparations exposed to smoked Hanks' solution for 1.5 hours and then challenged with bacteria in unsmoked Hanks' solution (Experimental Condition II). The data also demonstrates that smoked Hanks' solution previously used as a suspending medium for alveolar macrophages loses its ability to impair antibacterial activity. As shown in Table XIII (Experimental Condition III) macrophages challenged with bacteria in smoked Hanks' solution previously subjected to alveolar macrophage activity for 1.5 hours, phagocytized and destroyed staphylococci as effectively as control macrophages.

Conclusions: Smoked Hanks' solution caused a marked impairment of the antibacterial capacity of alveolar macrophages. This toxic effect is eliminated by subjecting smoked Hanks' solution to alveolar macrophage activity. It appears reasonable to assume that alveolar macrophages are capable of reducing or eliminating the water soluble constituents of the gas and/or particulate phase of whole cigarette smoke responsible for depressed antibacterial activity, in vitro. Whether this effect represents physical adsorption of smoke products on the surface of macrophages or phagocytic removal and ultimate detoxification of smoke products by alveolar macrophages remains unclear.

(C) The Influence of Serum Factors on Macrophage Function During Smoke-Exposure: For this purpose, alveolar macrophages harvested from untreated rabbits were incubated in tissue culture flasks containing Hanks' solution with and without 0.1% glucose containing various concentrations of normal rabbit serum. Cigarette smoke from a commercial brand of non-filtered cigarettes was introduced immediately after bacterial challenge (*S. aureus*). As shown in Table XIV, the efficacy of macrophage function during smoke-exposure is influenced by the concentration of serum in the suspending medium.

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In Hanks' solution (0.1% glucose) containing 5.0% serum, the antibacterial activity of alveolar macrophages was not adversely affected by introducing 1, 2, 4 and 8 ml. of whole cigarette smoke immediately after bacterial challenge. However, at a serum concentration of 2.5%, 8 ml. of smoke suppressed antibacterial activity. Under culture conditions in which the serum concentration was reduced to 1.0% and 0.5% the antibacterial activity of macrophages was reduced by the presence of 4 and 8 ml. of cigarette smoke. Studies in which the concentration of serum was varied have also been performed with Hanks' solution without glucose (Progress Report No. 1). Under these conditions, serum exerted the same influence on macrophage function during smoke exposure as noted above.

Conclusions: The efficacy of macrophage function during smoke-exposure was not affected by the absence or presence of added glucose in the culture medium but was influenced by the concentration of serum in the suspending medium.

(D) The Effect of Prior Exposure of Macrophages to Cigarette Smoke on Antibacterial Activity: To assess the effect of pre-exposure to whole cigarette smoke on macrophage function, macrophage cultures were exposed to cigarette smoke for 1.5 hours and then challenged with bacteria. Under these circumstances of smoke-exposure, studies were also performed to determine if changes in the antibacterial activity of smoke-exposed macrophage were reversible under in vitro conditions of recovery, namely, incubation of smoke-exposed tissue culture preparations in smoke-free environment prior to bacterial challenge. For this purpose, macrophage cultures were exposed to whole cigarette smoke for 1.5 hrs. At this time, the tissue culture flasks were flushed out with sterile room air and the culture media was removed with a pipette. Fresh media was then added to the tissue culture flask and the macrophage cultures were then incubated for 1.5 hours in the absence of smoke prior to bacterial challenge. The data presented in Tables XV and XVI clearly demonstrates that prior exposure to 8 ml. of whole cigarette smoke caused an impairment of phagocytosis and bacterial killing that was not reversed by introducing an interim period of incubation in a smoke-free environment prior to bacterial challenge.

Conclusions: Exposure of alveolar macrophages to whole cigarette smoke under in vitro conditions of smoke-exposure caused an irreversible impairment of phagocytic and bactericidal powers of alveolar macrophages.

(E) The Effect of Multiple Washings of Smoke-Exposed Alveolar Macrophages on Cigarette Smoke Induced Changes in Antibacterial Activity: Studies of the reversibility of the adverse effect of cigarette smoke on macrophage function were performed under in vitro conditions of recovery. The macrophage cultures were first exposed to cigarette smoke for 1.5 hours. At this time, the tissue culture flasks were flushed out with sterile room air, the culture media removed and the adhering macrophages washed 3 additional times with Hanks' solution. Fresh Hanks' solution was then added to the tissue culture flasks and the macrophage cultures challenged with *S. aureus* for 1.5 hours in the absence of cigarette smoke.

The data presented in Table XVII clearly demonstrate that prior exposure to cigarette smoke caused an impairment of phagocytosis and bacterial killing that was partially, but not totally, reversed by multiple washing of smoke-exposed macrophage cultures. As shown in Table XVII, compared to appropriate controls, the percent bacteria killed by unwashed and washed smoke-exposed macrophages was decreased 29% and 19%, respectively.

Conclusions: Multiple washing of alveolar macrophages after smoke-exposure reduces but does not totally reverse the adverse effect of cigarette smoke on macrophage function.

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(F) The Effect of Filtered Cigarette Smoke on the Antibacterial Activity of Alveolar Macrophages: Studies were performed with macrophage cultures exposed to whole cigarette smoke drawn through a glass fiber filter disc**. The filtered smoke was introduced immediately after bacterial challenge and maintained for 1.5 hours. As shown in Table XVIII, 8 ml. of filtered cigarette smoke caused less of an impairment of macrophage function than 8 ml. of whole smoke. In addition, 8 ml. of filtered cigarette smoke did not interfere with phagocytosis but impaired intracellular destruction of ingested bacteria. The data also indicates that 16 ml. of filtered smoke were required to produce the same interference of phagocytosis and bacterial destruction caused by exposure of macrophage cultures to 8 ml. of whole smoke.

Conclusions: Removal of the particulate phase of whole smoke reduced the toxic effect of cigarette smoke on the antibacterial activity of alveolar macrophages. The particulate and gas phases of whole smoke may have distinct effects on macrophage function.

IV. The Influence of Extended Exposure to Puffed Cigarette Smoke on Pulmonary Defense against Inhaled Staphylococcus aureus under In Vivo Conditions of Bacterial Challenge and Smoke-Exposure:

(A) The Effect of Cigarette Smoke on Lung Clearance: For this purpose experimental and control mice were first exposed to cigarette smoke and a secondary air flow of room air and then challenged with aerosols of Staphylococcus aureus for 30 minutes. Immediately after aerosol exposure groups of smoke-exposed and control mice were sacrificed to determine the number of bacteria deposited in their lungs. The remainder were killed 1 hour and 4 hours after bacterial challenge to ascertain the number of viable bacteria remaining in the lungs of smoke-exposed and control mice. These studies were performed immediately after daily exposure to cigarette smoke for 1 hour for 3, 5, 10 and 15 days. As shown in Table XIX, exposure to cigarette smoke for 15 days caused an impairment of 1 hour clearance; control animals cleared 48% of the bacteria and smoke-exposed 28%. In contrast, the 4 hour clearance rates of control and smoke-exposed were comparable; 84% and 82%, respectively. Comparable results were obtained in studies in which mice were exposed to cigarette smoke for 3, 5 and 10 days (Tables XX - XXII).

Conclusions: Exposure to Cigarette Smoke caused an impairment of bacterial clearance that was reversible within hours:

(B) The Effect of Cigarette Smoke on Basal Macrophage Yields: The effect of cigarette smoke on the numbers of alveolar macrophages harvested from the lungs of mice was studied by comparing the macrophage yields obtained from mice exposed to a secondary air flow (Control Group) and mice challenged daily for 1 hour with puffed cigarette smoke for 15 days. The data presented in Table XXIII demonstrates that smoke inhalation provoked a 2.6 fold increase in macrophage numbers and did not adversely effect cell viability (Table XXIV).

Conclusions: Smoke inhalation caused a marked increase in basal macrophage numbers and did not adversely effect cell viability.

(C) The Effect of Cigarette Smoke on the Mobilization of Alveolar Macrophages in Response to a Bacterial Challenge: These experiments were performed with control and experimental mice exposed to a secondary airflow and puffed cigarette smoke daily for 1 hour for 15 days. Basal macrophage yields were obtained from groups of control and smoke exposed mice and the remainder were exposed to an aerosol of S. aureus for 30 minutes. Immediately after aerosol exposure separate groups of control and smoke-exposed animals were sacrificed to determine bacterial deposition and mobilized macrophage

** Cambridge glass fiber filter, Phipps and Bud, Richmond, Va.

yields. As shown in Table XXV, the mobilized macrophage yields obtained from control and smoke exposed animals immediately after bacterial challenge were comparable.

Conclusions: Extended exposure to cigarette smoke did not mitigate the mobilization of alveolar macrophages in response to a bacterial challenge.

(D) The Effect of Cigarette Smoke on the Antibacterial** Activity of Alveolar Macrophages: In order to compare the antibacterial activity of smoke-exposed and control rabbits, experimental and control animals were exposed daily for 1.0 hour to cigarette smoke and a secondary air flow, respectively, for 15 days. Alveolar macrophage harvests from both categories of animals were treated as follows: washed five times with Hanks' solution, placed in tissue culture flasks containing Hanks' solution and 2.5% normal rabbit serum and challenged with *S. aureus*. As shown in Table XXVI, alveolar macrophages harvested from smoke-exposed macrophages phagocytized and destroyed staphylococci as effectively as macrophages from control animals.

Conclusions: The antibacterial activity of alveolar macrophages harvested from smoke-exposed animals and washed several times prior to bacterial challenge was unimpaired.

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** % changes in antibacterial activity of smoke-exposed macrophages derived as follows: % phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

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9. Details of Experimental Design and Procedures: (Cited references on pages 2A-31 to 2A-33).

(I) Methods of Procedure:

A. Smoke-Exposure System: A smoke generating apparatus is used to deliver puffed cigarette smoke under controlled conditions. In the studies proposed herein, the apparatus will be adjusted to deliver a 35 ml puff of 2 seconds duration from a commercial brand of non-filtered cigarettes. The time interval between puffs from a single cigarette will be set at 58 seconds. The instrument is equipped with a rotating disc which can accommodate 30 cigarettes at one time, so that it is possible to maintain a continuous stream of puffed smoke generated at a rate of 1 L/min. The initial smoke delivered by the cigarettes is diluted and transported across an animal exposure chamber by a secondary air flow. Control animals are placed in a plexiglass chamber and exposed to a secondary air flow. These are the same conditions of smoke exposure used to assess the influence of acute and extended exposure to puffed cigarette smoke on bacterial clearance and alveolar macrophage function in studies reported under Supporting Data, pages 2A-7 to 2A-8. In studies with filtered cigarette smoke, the smoke generating apparatus will be modified to include the insertion of a glass fiber filter disc*. A gas partitioner will be used to monitor the total hydrocarbon content in the smoke-exposure chamber. The tar concentration of smoke will be determined by established methods. Air samples from the smoke exposure chamber will be collected on filter paper, weighed, extracted in alcohol and subjected to spectrophotometric assay.

B. Conditions of Animal Smoke-Exposure: Animals will be exposed daily for 1.0 hour for 3 weeks to whole cigarette smoke or cigarette smoke passed through a glass fiber disc. The maximum number of total days of smoke-exposure planned over the entire 3 week study period is 15 days. However, total days or daily length of smoke-exposure will be reduced if toxicity, animal death or bacterial contamination of broncho-pulmonary tissue are noted. The daily length of exposure corresponds to the smoke-exposure periods previously used to study the effect of acute exposure to cigarette smoke tracheal clearance, lung clearance and alveolar macrophage activity. Under these conditions, it will be possible to determine the effect of whole and filtered cigarette smoke on pulmonary defense and to correlate these observations with the data obtained to date under conditions of acute and extended exposure to cigarette smoke (see Supporting Data, pages 2A-3 to 2A-5). The proposed protocol is similar to that used by LaBelle et al (1) to study the effects of acute and extended exposure to cigarette smoke on pulmonary clearance of radioactive test particles.

C. Bacterial Aerosol Exposure Unit: Since bacterial clearance studies comprise a major part of the proposed research project, a review of the method of aerosol formation, exposure and quantitative aspects of bacterial clearance is indicated. Aerosols are generated from a buffered suspension (pH 7.3) of staphylococci contained in glass nebulizers. The initial spray from the nebulizers is directed into mixing chambers through which a secondary air flow of 100 cubic feet/min is drawn. The large volume of secondary air serves to mix, dilute and dry the initial bacterial aerosol; direct it past an interposed baffle for removal of large droplets; and then carry it through a large plexiglass exposure chamber that can accommodate up to 200 mice. The particle size distribution of the bacterial aerosol is monitored with an Andersen Sampler (2). White male Swiss Webster mice are divided into groups of 5 to 10 animals and exposed to Staphylococcal aerosols for 30 min. Immediately after exposure (0 time) one group of animals is sacrificed to determine the numbers of viable bacteria deposited in the trachea and lungs, respectively. The trachea and lungs are removed as separate blocks and individually ground in glass homogenizers. The remaining groups

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of challenged mice are killed and processed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after aerosol exposure. Bacterial counts are obtained from nutrient agar pour plates of lung and tracheal tissue homogenates. By this method paired studies of lung and tracheal clearance of bacterial deposits are possible. By subtracting the mean number of culturable bacteria retained in the lung at each interval during the post aerosol exposure period from the number originally deposited (0 time), the mean number of staphylococci cleared by the lung may be derived. From these data lung clearance rates are calculated by expressing the number retained in the lung as a percentage of the deposition number (0 time) and subtracting this value from 100%. Similarly, from the numbers of bacteria present in the trachea at 0 time and at the same intervals after aerosol exposure, the numbers and percent bacteria cleared by the trachea may be obtained.

D. Harvesting of Alveolar Macrophages: In order to correlate macrophage activity with bacterial clearance, a method has been developed in this laboratory for harvesting alveolar macrophages from the murine lung (3,4). Mice are sacrificed and the trachea and lungs exposed. The intact lung is washed by 5 successive 1.0 ml washes with Hanks' solution and the harvested cells recovered by centrifugation. By this method, it is possible to obtain 1.0 to 2.0×10^7 macrophages from each mouse's lung with 90% viability. Total cell counts are performed in a bright line hemocytometer and differential counts are made on Wright stained smears. Cell viability is assessed by the capacity of alveolar macrophages to reject the stain Eosin I. The number of alveolar macrophages that are available in harvest by this technique under basal conditions is referred to as a basal yield. Therefore, mobilization is taken to represent the increase in macrophage numbers over basal levels harvestable from the lungs after a bacterial challenge. The mobilization of alveolar macrophages may be quantitated by this technique. Studies completed in our laboratory to date indicate that macrophage yields are increased 1.5 times basal levels after exposure to aerosols of a phosphate buffer or dead *Staphylococcus aureus* (4), and 2 to 3 times basal levels in response to aerosols of viable *S. aureus* (3,4). During the post-aerosol exposure period there is an initial drop in macrophage numbers but elevated levels are restored in 30 min. and this increase is maintained for 4 hours (4). This sustained response is blocked by alcohol, but not by acute exposure to cigarette smoke (4,3) (See Supporting Data, pages 2A-3 to 2A-5).

Alveolar macrophages are harvested from the lungs of albino rabbits weighing 1.0 to 2.0 kilograms by the general method of Myrvik et al (5). The animals are killed by injecting air in the marginal ear vein. This method of sacrificing animals is used to avoid any depressant effects that anesthetics may have on alveolar macrophage function. The trachea and lungs are exposed, and macrophages harvested by washing out the intact lungs with 17 ml. of Hanks' solution. The harvested cellular contents of the lungs are recovered by centrifugation at 2000 rpm for 20 minutes. Total cell counts are performed in a bright line hemocytometer, and differential counts are made on Wright stain smears. By this method, 95% of the macrophages harvested are viable as determined by the Eosin I technique (6).

E. Acellular Fractions of Lung Harvests: Recent studies suggest that secreted fluids present in the bronchopulmonary tree may independently or in concert with alveolar macrophages play a significant role in pulmonary defense against inhaled bacteria. In this regard, alveolar living material (ALM) has been reported to enhance the bactericidal activity of rat alveolar macrophages (7). Secretory IgA is present in the tracheobronchial washing of normal patients (8) and has the capacity to kill *Escherichia coli* (9) and inhibit the adherence of certain strains of *Streptococci* to epithelial tissue (10). For these reasons, the acellular fractions of lung harvests (ACF) will be collected, concentrated, Secretory IgA levels monitored and ALM isolated.

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In addition, the antibacterial and phagocytosis properties of concentrated acellular fraction and AIM against *S. aureus* will be assessed in the phagocytosis system detailed on page 2A-9. These studies will be performed with acellular fractions of lung harvests obtained from control and smoke-exposed animals.

The method of LaForce et al (7) will be used to isolate alveolar lining material from the lungs of rabbits. Rabbits are sacrificed by injecting air in the marginal ear vein and the trachea and lungs are exposed. The trachea is cannulated with a sterile polyethylene tube, and (0 ml of sterile heparinized saline (10 units per ml) are introduced in the lung and recovered by aspiration. The recovered bronchoalveolar saline lavage fluid is centrifuged at 800 g for 8 minutes; the supernatant decanted and saved. The cell free supernatant is centrifuged at 40,000 g at 4°C for 20 minutes. The recovered precipitated pellet represents the alveolar lining material or surfactant fraction (11).

In order to study, the antibacterial and phagocytosis promoting characteristics of the entire acellular fraction of lung harvests, bronchoalveolar saline lavage fluid recovered from rabbit lungs will be initially concentrated by ultrafiltration (Diaflow Membranes, Amicon Corp) and fractionated by gel column chromatography.

The presence of Secretory IgA (SIgA) in bronchoalveolar washings will be monitored by double immunodiffusion (12) against anti-SIgA and anti-secretory piece sera. SIgA levels will be quantitated by single radial immunodiffusion (13) utilizing anti-SIgA serum impregnated in the agar gel and SIgA as the antigen standard. The SIgA antigen standard will be prepared from clarified colostrum (14). The latter procedure includes: (a) separation by gel chromatography and further purification by anion-exchange chromatography using a stepwise elution gradient of phosphate buffers of varying ionic strengths. The purity of SIgA will be assessed by disc electrophoresis (15).

(II) Details of Experimental Design of Proposed Research Plan: In keeping with the intended purpose of the proposed research, a series of studies are planned to evaluate the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance of bacterial deposits. All studies will be performed under in vivo conditions of smoke-exposure.

A. Paired Observations of Tracheal and Lung Clearance: For this purpose, experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with aerosols of *S. aureus* for 30 minutes. Immediately following aerosol exposure (0 time) some of the smoke-exposed and control animals will be sacrificed to determine the number of bacteria initially deposited in their trachea and lungs. The remainder will be killed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after bacterial challenge to ascertain the number of viable bacteria remaining in the trachea and lungs of smoke-exposed and control mice. From this data, it is possible to calculate the rate at which bacteria are cleared from the trachea and lungs. These studies will be performed in mice exposed daily for 1.0 hours to cigarette smoke over a 3 week period. Tracheal and lung clearance rates will be measured immediately following and 24 to 48 hrs after daily exposure to cigarette smoke for 15 days. The following specific information is sought from these studies: (a) the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance of bacterial deposits and (b) the reversibility of any adverse change in clearance rates attributable to smoke-exposure. Since this aspect of the proposed research will be completed by the end of the current grant support, a limited number of clearance studies will be performed to audit the conditions of smoke exposure

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established in the alveolar macrophage studies detailed in the current grant proposal and to correlate the data obtained from these observations with the clearance and macrophage data compiled to date (See Supporting Data, pages 2A-3 to 2A-5).

B. Alveolar Macrophage Activity: These studies will be performed under the same conditions of smoke-exposure used to assess the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance. In this regard, mice and/or rabbits will be exposed daily for 1.0 hrs to cigarette smoke over a 3 week period and alveolar macrophage activity will be assessed immediately following and 24-48 hrs after daily exposure to smoke for 15 days.

(1) Phagocytosis and Intracellular Killing Activity: Studies will be performed to determine the effect of extended exposure to whole and filtered cigarette smoke on the antibacterial activity of alveolar macrophages in the intact and functioning lung. For this purpose, large numbers of experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with staphylococcal aerosols for 30 minutes. Immediately after bacterial challenge (0 time) and at hourly intervals during a 4 hour post-aerosol exposure period, alveolar macrophage harvests obtained from smoke-exposed and control animals are processed as follows: bacterial counts are obtained from an aliquot of the total lung washout and the remainder is separated by differential centrifugation at 1500 rpm for 15 min into a supernatant fraction containing free bacteria and a cellular fraction laden with alveolar macrophages and phagocytized bacteria. The number of viable bacteria present in each fraction is determined by a standard pour plate technique. The decrease in the number of viable bacteria present in the total lung washout, as a function of time, is used as an index of the clearance rate of the lung sample obtained by the lavage technique. Similarly, a decrease in the viable counts of the supernatant and cellular fractions as a function of time, are used as a measure of the quantitative phagocytic and intracellular killing activity, respectively, of alveolar macrophages. These studies will be performed under the same conditions of smoke-exposure as used to assess the effect of extended exposure to whole and filtered cigarette smoke on bacterial clearance. In this regard, antibacterial activity will be assessed immediately following and 24 hours after daily exposure to cigarette smoke for 15 days. Under these conditions it will be possible to compare the effect of whole and filtered cigarette smoke on the antibacterial activity of macrophages under in vivo conditions of bacterial challenge and to assess the relative influence of the particulate and gas phases of smoke on this important parameter of macrophage function. The protocol will also permit an assessment of the reversibility of any impairment in phagocytic and bactericidal powers that may occur as a result of exposure to cigarette smoke.

In a separate series of studies, alveolar macrophages harvested from smoke-exposed and control mice and rabbits will be challenged with *S. aureus* in an in vitro phagocytosis system. The methods, procedures and details of the in vitro phagocytosis are presented in detail in the Methods and Materials Section of Supporting Data, page 2A-9. Fixed numbers of staphylococci are added to known numbers of macrophages adhering to the flat surface of a tissue culture flask containing Hanks' solution (0.1% glucose). Immediately after bacterial challenge and at various intervals thereafter, the extracellular fraction containing free or unphagocytized bacteria is isolated by removing the supernatant fluid from macrophage cultures with a pipette. To recover the macrophage fraction, the tissue culture flask is vigorously shaken after adding glass beads and distilled water. Bacterial counts are obtained from each fraction by a standard pour plate technique. By this method, it is possible to determine the percent bacteria phagocytized, percent intracellular survival of phagocytized bacteria and

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percent bacteria killed by macrophages. The purpose of these studies is to obtain data that will permit a better understanding of the antibacterial activity of alveolar macrophages in the intact and functioning lung under normal conditions and during the inhalation of cigarette smoke. For this reason studies are planned to evaluate the influence of increased macrophage numbers, opsonization of bacteria, pre-treatment of bacteria with alveolar lung material, pre-treatment of bacteria with the total acellular fraction of lung harvests on the phagocytic and bactericidal powers of alveolar macrophages. In separate studies alveolar macrophages will be challenged with *S. aureus* and incubated at 37°C in tissue culture flasks containing Hanks' solution supplemented with the following: (a) normal serum with and without specific immune serum added, (b) alveolar lining material (ALM) alone, ALM plus normal and immune serum, ALM plus immune serum and ALM plus normal serum and (c) concentrated acellular fraction (CAF) alone, CAF plus normal and immune serum, CAF plus immune serum and CAF plus normal serum. This information is needed for proper analysis of the events observed under in vivo condition of an airborne bacterial challenge and is in keeping with recent observations that suggest local immune systems and pulmonary secretion play a significant role in pulmonary defense (7,8) and, as such, may act as mediators of alveolar macrophage function. In addition, the above information will be used to establish in vitro culture conditions necessary for meaningful studies of macrophage mobilization and the metabolic activity of alveolar macrophages outlined below; namely, in vitro culture conditions that best reflect the in vivo circumstances in the live and intact lung.

(2) In Vitro Studies of Cell Adhesiveness and the Motility and Migratory Response of Basal and Mobilized Alveolar Macrophages to Chemotactic Substances: For this purpose, a method of studying leukocyte motility reported by Carruthers (16) will be adapted to alveolar macrophages. This method is based on the ability of motile cells to move through the pores of a membrane filter. In this regard, two O-ring joints separated by a millipore filter are clamped together and sealed which results in the formation of 2 distinct chambers. Fixed numbers of alveolar macrophages ($1-3 \times 10^7$) suspended in Hanks' solution (3 ml.) containing glucose will be introduced into chamber number one which is then sealed with a paraffin plug. The second chamber is filled with 5 mg. of insoluble potato starch and plugged. After an initial period of incubation at 37°C to permit monolayer formation, the chambers are inverted so that the test cells are now on the bottom side of the filter, and the chemotactic substance, if present, is on the top side of the filter. The chamber is then placed in an incubator at 37°C for 4 hours. At the end of various hourly intervals, the filter is removed, stained with hematoxylin and subjected to microscopic study. In this way, the number of cells on both sides of the filter are enumerated and used as an index of the migratory response elicited by stimulatory agents. Separate studies are planned to evaluate the changes in cell adhesiveness that occur in phagocytizing alveolar macrophages. The method of studying the adhesive properties of blood leukocytes reported by Allison and Lancaster (17) will be adapted to alveolar macrophages. Test tube cultures of fixed numbers of lung phagocytes suspended in Hanks' solution will be challenged with known numbers of bacteria. Under conditions favoring maximum phagocytosis, microscopic methods will be used to determine the formation of cell aggregates that clump together by cultures of phagocytizing macrophages. Changes in cell adhesiveness will be evaluated under the same experimental conditions described above to assess motility.

An understanding of this aspect of alveolar macrophage activity is desirable because motility and changes in cell adherence may be critical steps in the mobilization and migration of lung phagocytes to pulmonary sites, as well as their subsequent antibacterial activity during the normal situation and in response to the

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inhalation of viable bacteria and cigarette smoke. The data obtained from these studies will be correlated with the results of studies completed in this laboratory concerning the mobilization of alveolar macrophages in response to the inhalation of viable bacteria in the normal situation and during the inhalation of cigarette smoke. (See Supporting Data, Results pages 2A-10 and 2A-15).

(3) Metabolic studies: The present grant will include studies of the energy metabolism, lipid compositions, hydrolytic enzyme activity and peroxidative metabolism of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. This information is desirable, since energy output and cell metabolism represent important potential links in the successful mobilization and subsequent antibacterial activity of alveolar macrophages. Indeed, alterations in hydrolytic enzyme activity and peroxidative metabolism may interfere with the primary immunological function of macrophages in pulmonary defense, namely, the destruction of inhaled microorganisms. In addition, interest in the lipid fraction of phagocytic cells resides in the fact that lipids represent a source of high energy metabolism and are intimately involved in membrane phenomena associated with phagocytosis. The metabolic studies will be performed under the same culture conditions (suspending medium) used to assess the influence of cigarette smoke on the phagocytic and bactericidal capacity of alveolar macrophages.

Respiration studies: Experiments are planned which will permit determinations of the oxygen consumption and lactic acid content of alveolar macrophages incubated in basal medium and media supplemented with glucose plus serum with and without the presence of particles that induce phagocytosis. In separate studies serum will be supplemented with and/or replaced by alveolar lining material and the concentrated acellular fraction of lung harvests as outlined in the phagocytosis studies detailed on page 2A-24. These studies will be performed with the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of a phosphate buffer for 30 minutes.

Measurements of oxygen uptake will be determined in a Gilson respirometer using flasks with a 15 ml capacity containing monolayers of alveolar macrophages in a total liquid volume of 3.2 ml (18). The CO_2 will be absorbed by 0.2 ml of 20% KOH in the center well. In accordance with the protocol of each study, glucose (5.0 to 10 mM) and polystyrene spheres at a concentration of 2.0 to 2.5 mg/ml will be introduced via the side arm. After completion of the oxygen consumption measurements, the cells will be harvested and their lactic acid content determined by the method of Barker et al (19). For this purpose, the recovered macrophages will be washed in saline and cell extracts are to be prepared as described by Myrvik et al (20).

Glucose metabolism studies: Experiments with specifically labelled glucose as substrate will be performed to evaluate the effect of cigarette smoke on the metabolism of glucose by alveolar macrophages. These studies will be done with the same categories of basal and mobilized macrophages used in the respiration studies.

The radioactive measurements will be done as described by Myrvik et al (20). Alveolar macrophages are harvested and placed in Erlenmeyer flasks containing medium 199 without serum or glucose. In separate experiments ECG and heat killed staphylococci will be added to all flasks except the control flasks. After incubation at 37°C in a shaker bath for 1, 2, 4, 6 and 18 hours, glucose 1- C^{14} or glucose 6- C^{14} is added to the flasks, and reincubated for 1 hour. The reaction is stopped with sulfuric acid and counts obtained with a liquid scintillation counter.

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ATPase Activity: Experiments are planned to compare the ATPase activity of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. ATPase activity will be determined from the liberation of inorganic phosphate (Pi) upon incubation of alveolar macrophages with adenosine triphosphate (ATP) as outlined by Wahler et al (21). The assay medium will contain 100 mM sucrose, 30 mM glycylglycine, 30 mM imidazole, 5 mM MgCl₂, 2 mM ATP, protein equivalent of cells 50 to 100 ug and, as indicated, 50 mM NaCl plus 5 mM KCl (pH 7.5). In studies performed in the absence of Na⁺ and K⁺, the medium will contain 200 mM sucrose. Reactions will be carried out in a 2nd volume for 20 minutes at 30°C and activity will be expressed as micromoles of Pi liberated per milligram of protein per hour.

Catalase Activity, peroxidase activity and hydrogen peroxide production: Recent studies (22) have presented evidence for the presence of a catalase-dependent peroxidative metabolism. Peroxidative metabolism represents a biochemical pathway capable of increasing glucose metabolism and hydrogen peroxide formation. In this regard, published reports clearly demonstrate that phagocytosis by normal alveolar macrophages is accompanied by increased glucose metabolism (35) and intracellular recovery of hydrogen peroxide (22). The potential role of catalase controlled concentrations of hydrogen peroxide as an intracellular bactericidal agent has been recognized. For these reasons studies are proposed to compare the catalase activity, peroxidase activity and hydrogen peroxide production in macrophages harvested from control and smoke-exposed rabbits. These studies will be performed with basal macrophages, and macrophages mobilized in response to the inhalation of heat-killed staphylococci harvested from both control and smoke-exposed animals.

Catalase activity will be determined by the method of Feinstein (23) using 0.1M sodium perborate as substrate. Perborate utilization in 5 min is measured by titration with a 0.1N solution of potassium permanganate after the reaction is stopped with 1N sulfuric acid. Catalase activity will be determined after incubation in Krebs Ringer Phosphate Solution containing 15% homologous serum and 5.5 mM glucose at pH 7.4. Measurements will be performed on macrophage preparation after disruption by either homogenization or repeated freeze-thawing using acetone-dry ice. Both total extract and supernatant obtained by centrifugation at 8000 rpm for 10 min will be assayed. Activity will be expressed as milliequivalents of perborate utilized in 5 min, 1 U representing the utilization of 1 mEq of perborate. Peroxidase activity will be assessed by a modification of the guaiacol method of Chance and Maehly (24). Whole extracts of freeze-thawed cells will be employed. The assay medium contains 0.1 M phosphate buffer at pH 7.4, 0.5 ml of 100 mM guaiacol, 0.2 ml of extract sample, and 0.02 ml of ice-cold 10 mM H₂O₂. Absorbancy changes due to tetraguaiacol formation will be measured at 750 nm in a spectrophotometer and the time required to produce an 0.05 U increase in absorbancy recorded. Results will be expressed in reciprocal seconds per 10⁹ cells.

Hydrogen peroxide will be determined spectrophotometrically on dialysates of AM as described by Paul and Shara (25). The nonfluorescent dye, diacetyl-2,7-dichlorofluorescein (LDACDF), was synthesized by the method of Brandt and Keston (26) and the fluorescence of the oxidized product of alkali-activated LDACDF was measured with an Aminco-Bowman spectrofluorimeter. The excitation wave length was 340 nm and the emission wave length 525 nm.

Hydrolytic enzyme studies: The present grant proposal will include a study of the enzymatic activity and intracellular distribution of a specific group of hydrolytic enzymes in basal and mobilized macrophages harvested from control and smoke-exposed animals. The enzymes acid phosphatase, lysozyme, lipase, beta-glucuronidase and cathepsin are of immediate interest because their activity is increased

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in the ECG induced alveolar macrophage (17, 28-29). To accomplish these aims, the enzymatic activity detectable in the cell free supernatant fraction and alveolar macrophage fraction of lung harvests will be assayed. Interest in both fractions of lung harvests resides in the fact that bronchial mucus contains several poorly defined substances (30) including lysozyme (31) that exert nonspecific bacteriostatic and bactericidal activity against gram positive and gram negative bacteria as well as the potential phagocytosis promoting factor present in pulmonary secretions (7,8). In addition, alveolar macrophages by virtue of their high hydrolytic enzyme content, presence in large numbers and rapid turnover rate may contribute to the enzymatic activity found in mucus secretions.

A comparison will be made of the enzymatic activity and intracellular distribution of the above hydrolases in the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of viable or heat-killed staphylococci. For this purpose, smoke-exposed and control animals will be exposed to the bacterial aerosols for 30 minutes. Enzyme determinations will be made with the lung harvests obtained from both groups of animals immediately after bacterial challenge and at hourly intervals over a 4 hour post-aerosol exposure period. The choice of laboratory animals (mice or rabbits) to be used in all enzyme studies will be governed by the minimal yields necessary for adequate enzyme assay. The lung washing obtained from animals by the lavage technique will be separated into a cell-free supernatant fraction and cellular fraction containing alveolar macrophages by centrifugation at 4°C for 15 min at 10,000 rpm (31). The supernatant will be frozen at -60°C and stored until assay. The harvested alveolar macrophages will be washed twice in phosphate buffered saline (pH 7.2); quantitated by hemocytometer count; and viability assessed. Saline extracts of the washed cells are to be prepared for enzyme activity studies as outlined by Myrvik et al (20) cell disruption by alternate freezing and thawing for 5 consecutive cycles and removal of cellular debris by centrifugation at 2,500 rpm for 10 min at 4°C. The completeness of cell disruption will be audited by phase optics. In order to evaluate the contribution made by alveolar macrophages to the enzymatic activity detectable in the supernatant fraction, in vitro studies of the rate of release of the enzymes in question by pulmonary macrophages are planned. As outlined by Holzman et al (31) large numbers of alveolar macrophages will be suspended in tissue culture medium and samples of the cell population assayed for specific enzymatic activity after incubation at 37°C for various periods of time. In studies of the intracellular distribution of the hydrolases, alveolar macrophages will be suspended in a 0.25M sucrose solution, ruptured by homogenization and sedimented by centrifugation into four fractions (27). The nuclear fraction (N) will be sedimented by centrifugation at 250 X G; the heavy granule fraction (HG) at 5000 X G for 15 min; and the light granular fraction (LG) and supernatant fraction (S) by centrifugation at 25,000 X G. Each fraction will be submitted to 5 cycles of freezing and thawing and clarified by centrifugation at 2500 X G for 20 min and subjected to enzyme assay.

For the purpose of enzyme analysis, lysozyme will be quantitated by using suspensions of *Micrococcus loidodykticus* as substrate. Tests will be standardized with known amounts of crystalline egg white lysozyme and results expressed as egg white lysozyme equivalents. Acid phosphatase will be measured by the procedure of Hofstee (32) using O-carboxyphenyl phosphate as substrate. An increase in absorbance of 0.001 optical density units/min under standard conditions will be taken to represent a unit of activity. Beta-glucuronidase will be assayed by the procedure of Fishman et al (33) using phenolphthalein mon-Betaglucuronide as substrate with reactions carried out in 0.1M acetate buffer at pH 4.5 and 38°C. One unit of glucuronidase is

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the activity resulting in the liberation of 1 $\mu\text{g/hr}$ phenolphthalein. Lipase activity will be determined as outlined by Cohn et al (28) in which naphthol laurate serves as substrate and the increase in activity caused by sodium taurocholate is taken to represent minimal lipase activity. The results are to be expressed as micromoles of naphthol liberated per hour. Cathepsin will be assayed employing a 2% solution of denatured hemoglobin as a substrate, as described by Anson (34) and protein digestion estimated with a spectrophotometer by absorption at 280 nm (27). A unit of activity being defined as an increase in optical density produced by 0.001 meq of tyrosine.

Lipid composition of basal and mobilized alveolar macrophages: The specific information sought from these studies in the qualitative and quantitative changes in the major lipid classes and fatty acid composition of alveolar macrophages associated with mobilization and phagocytosis in the normal activation and during the inhalation of cigarette smoke. To accomplish these aims a series of in vivo and in vitro studies are planned.

A comparison will be made of the lipid and fatty acid components extractable from alveolar macrophages harvested from unchallenged animals and animals exposed to aerosols of *S. aureus*. Alveolar macrophages will be harvested from the challenged animals immediately after aerosol exposure and at hourly intervals thereafter over a 6 hr. period. Since the presence of bacteria within alveolar macrophages may influence the outcome of these studies, appropriate controls will be used; namely, cells provoked by inert particles. In addition, *S. aureus*, in numbers equivalent to those deposited in the lungs of the test animals, will be carried through the same lipid extraction and analysis. These studies will be repeated with animals which have been exposed to cigarette smoke and cigarette smoke plus bacterial aerosols. A series of studies will be undertaken to determine the effects of metabolic inhibitors on the lipid composition of basal and phagocytizing alveolar macrophages. The specific information sought from these studies is as follows: (a) insight into the energy-yielding metabolic pathways utilized by alveolar macrophages during phagocytosis and (b) the correlations that exist between the effects of metabolic inhibitors on phagocytosis and the lipid composition of these cells. The rationale behind this approach resides in the observation that alveolar macrophages undergo a minimal increment in metabolic levels (O_2 uptake and carbohydrate metabolism) during phagocytosis. It is therefore proposed that an end result of metabolic activity, namely lipid composition, be studied. The specific metabolic inhibitors to be used are those which block glycolysis (monochloroacetate, sodium fluoride) Krebs' cycle activity (sodium malonate, sodium fluoroacetate) and respiratory inhibitors cyanide, (dinitrophenol and anaerobiosis) and others as indicated by the initial results observed. The concentrations of inhibitors to be used will be determined empirically as those concentrations which interfere with phagocytosis and alter lipid composition. However, the concentrations used by Oren et al (18), Karnovsky et al (35) and Ouchi et al (36) in evaluating the effect of these metabolic inhibitors in the metabolic levels of phagocytizing cells will be referred to. The protocol to be followed in the in vitro studies consist of incubating known numbers of alveolar macrophages in a balanced salt solution containing glucose, in tissue flasks at 37°C until monolayer formation occurs. At this time, polystyrene spheres (2.0 to 2.5 $\mu\text{g/ml}$, final concentration) and specific concentrations of metabolic inhibitors will be added to the monolayers. The mixture will be further incubated for 1 hour and harvested. An aliquot will be used for microscopic evaluation of phagocytosis and the remainder will be subjected to lipid analysis. The choice of laboratory animals to be used in all lipid studies will be governed by the minimal yields necessary for adequate lipid analysis. Total lipid extraction will be carried out by the method of Folch et al (37) which consist

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of multiple additions of chloroform methanol mixtures to washed cells incubated at 55°C for 4 hrs. The separation of lipid classes will be performed by the techniques of Fillerup and Mead (38) which consists of liquid-solid chromatography on silicic acid. Separation of the various lipid classes (sterol esters, tri-glycerides, free sterols, free fatty acids and phospholipids) is achieved by a step-wise application of eluants of varying polarity. Thin layer chromatography will be used to assess the purity of each fraction eluted by column chromatography and to identify the different lipid classes present in total lipid extracts. The technique to be used is that of Malins and Mangold (39) in which thin layer plates coated with silica gel G are spotted with lipid material and developed in polar and non-polar systems. The resolved lipid spots are then visualized with an aqueous solution of rhodamine G or iodine vapors. The fatty acid composition of alveolar macrophages will be studied by converting the fatty acids collected by column chromatography and those present in total lipid extracts to their respective methyl esters and employing gas liquid chromatography.

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LA-31
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KA-22
2A-33
References to Experimental Design

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Microbiology Research Laboratories are located as a complex in the Triboro Hospital at Queens Hospital Center, Jamaica, New York. They include: (a) an aerosol exposure laboratory, (b) a smoke-exposure laboratory, (c) individual laboratories for macrophage studies and microbiology, (d) 2 rooms for storage space and refrigerators. Animal quarters are provided in another area of the hospital. The entire laboratory area occupies approximately 700 square feet.

The equipment in this area includes a complete bacterial aerosol generating and exposure system with mixing chambers and decontaminating units, and an Anderson apparatus for measuring particle size of bacterial aerosols: A cigarette smoke generating apparatus and exposure chamber and a sequential sampler and gas liquid chromatographer unit for determining concentration of the particulate and gas phase of cigarette smoke. Other major instrumentation present include the following: (a) standard microscope, (b) 1 infusion pump, (c) a refrigerated centrifuge (d) 2 large refrigerators, (e) sonic dismembrator, (f) 2 water bath and shaker, (g) 1 freezer, (h) incubator and 1 environmental chamber, (i) 4 vacuum pumps, (j) pH meter, (k) Beckman DU2 recording spectrophotometer, (l) spectronic 20 spectrophotometer, (m) a Gilson respirometer and (n) a lyophilizer unit. There is also equipment for qualitative and quantitative bacteriology studies, tissue homogenization, administration of gas mixtures and animal surgery. A Revco deep freezer (-75°C) is also available. In addition, high performance scintillation counters capable of isotope work are present in the hospital and available for research use.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append): See attached Curriculum Vitae.
See pages 6 to 9.13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See pages 10 to 11.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

Joseph J. Guarneri, Ph.D.
Principal Investigator

hrs/wk

% time

Amount

20 hrs./60 hrs.

None

Marcelino Sierra, Ph.D.

7 hrs.

None

Research Associate, Ph.D.
(to be recruited)

100%

15,000

Fringe Benefits

2,550

Technical

Amar Sarwal, M. S.

100%

11,288

1,919

Research Lab Aide

25%

2,200

374

Sub-Total for A

33,331

B. Consumable supplies (by major categories)

See page 4A for detailed list

Sub-Total for B

6,150

C. Other expenses (itemize)

Travel 700

Publications 150

Sub-Total for C

850

Running Total of A + B + C

40,331

D. Permanent equipment (itemize) See page 4A for justification

1. Sorval RC 2-B Automatic Superspeed

Refrigerated centrifuge including rotors and heads (\$1000)

2. Gel Electrophoresis Cell (Bio Rad) with Power Source (\$555).

3. Fraction Collector - Isco Model 1200 pup (\$600)

4. LKB Peristaltic Pump (\$300)

Sub-Total for D

5,455

E

6,019

Total request

51,835

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|-----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | \$35,831* | 6,150 | 850 | 1,000 | 6,425 | 50,256 |
| Year 3 | 38,518* | 6,150 | 850 | - | 6,828 | 52,346 |

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B. Consumable Supplies

| | |
|--|---------|
| 1. Cigarettes for smoke studies | \$1,750 |
| 2. Mice Caesarian delivered | 1,000 |
| 3. Rabbits | 1,200 |
| 4. Immunodiffusion Plates, Anti SIgA and Anti Secretory Piece Sera, Radial Immunodiffusion kit and templates | 200 |
| 5. Chromatography columns with 4 way valve, accessories, and Reagents for Chromatography, Sephadex G 200, Aerylamide kit, DEAE | 353 |
| 6. Reagents and curvettes for enzyme studies and flasks and accessories for respiration studies | 500 |
| 7. Tissue culture glassware, tissue culture media and bacteriology media | 647 |
| 8. Isotopes uniformly labelled glucose - 1-C ¹⁴ and glucose 6-C ¹⁴ | 300 |
| 9. Petri dishes and plastic disposables | 200 |
| Total | \$6,150 |

D. Permanent Equipment (Justification)

1. The Sorval RC 2-B Superspeed Refrigerated Centrifuge is needed for the alveolar lining material studies (ALM) proposed on pages 2A-22 and 2A-25 of grant request. The instrument and its rotors permit centrifugation up to 49,500 g with controlled temperature. The latter permits g forces and controlled conditions necessary for the isolation of ALM without the loss of biological activity.

2. Gel Electrophoresis Cell with a power source is needed for the purification and characterization of Secretory IgA and other proteins of immunological interest in the acellular fraction of lung harvests. See pages 2A-23 of the grant proposal for specific studies in which Gel Electrophoresis will be used.

3 & 4. Fraction collector and LKB Perisaltic pinup are needed for the purification and isolation of proteins in the acellular fraction of lung harvests and lipid studies (page 2A-23 and page 2A-30).

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--|----------|--------------------|
| The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophages and Mucociliary Function. | The Council for Tobacco Research, U.S.A. Grant Nos. 547C, 547CR-1 and 547 CR-2. | \$63,152 | 7/1/71 to 6/30/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--|----------|--------------------|
| Important Determinants to Pulmonary Resistance | Long Island Jewish-Hillside Medical Center (approved) | \$36,340 | 7/1/74 - 6/30/76 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to:

Long Island Jewish-Hillside Medical Center

Mailing address for checks

Mrs. Eva Meyer, Grant Manager
Long Island Jewish-Hillside Medical Center
New Hyde Park, New York 11040

Principal investigator

Typed Name Joseph J. Guarneri, Ph.D.Signature Joseph J. Guarneri Date 1/14/74

Telephone 212-990-2335
 Area Code Number Extension

Responsible officer of institution

Typed Name Harold LightTitle Deputy Director

Signature _____ Date _____

Telephone 212 343-6700 2723
 Area Code Number Extension

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#9084 - LEADS

1003540062

February 12, 1974

Grant application No. 908M

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Gardner, Liebow and Wyatt

Subject: Sanford E. Leeds, M.D., Mount Zion Hospital and Medical Center,
San Francisco
Modified application No. 908M
"Role of the pulmonary lymphatics in the absorption of inhaled
particles and gases."

History

Application No. 908, in the amount of \$56,485 plus two additional years, was not recommended for funding. This proposal was exceedingly brief.

Request

Application 908M requests \$44,134 plus two additional years.

Documents Submitted

Attached is application dated Jan. 3, 1974 (20 pages).

Reprints of the three publications checked on page 20 of the application are available and will be sent if you wish.

Comments

To refresh recollection we enclose copies of the following:

1. Dr. Gardner's memo, site visit on July 11, 1973.
2. Dr. Loosli's memo, October 29, 1973.

FWN:gh
Encls.

FWN
F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 1 1974

Date: Jan. 3, 1974.

1. Principal Investigator (give title and degrees):

Sanford E. Leeds, M.D.

Asst. Clin. Professor of Surgery, University of California Medical School

Director, Experimental Surgery Laboratory, Mount Zion Hospital & Medical Center.

2. Institution & address:

Mount Zion Hospital and Medical Center

P. O. Box 7921

San Francisco, California 94120

3. Department(s) where research will be done or collaboration provided:

Experimental Surgery Laboratory

4. Short title of study:

Role of the pulmonary lymphatics in the absorption of inhaled particles and gases.

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: The route of absorption and lung clearance of inhaled particles has been extensively studied (1,2,3,4). However, there are few studies of the role of the pulmonary lymphatics in the absorption process. The parenchyma of the lungs have an extensive network of lymphatics which drain lymph toward the hilar lymph nodes and thence into the venous circulation mainly via the right lymphatic duct (RD). The role of lymphatics in the transport of particles from the alveoli is not well understood because the lymphatic and respiratory systems are difficult to study both anatomically and physiologically. Morrow (5) in a recent review states "few experiments either have been designed or have attempted to quantify the role of pulmonic lymphatics in dust removal, and even fewer have tried to elucidate the mechanisms and pathways of lymphatic uptake and transport". The specific aims of the research to be described is to elucidate the role of the pulmonary lymphatics in the clearance of inhaled particles from the alveoli and interstitial tissue of the lung.

The specific objectives are threefold:

- (1) To administer aerosols containing particles of different characteristics and to quantitate their appearance in pulmonary lymph proximally and distally to the hilar lymph nodes. The particles to be studied are in the range of 0.5 - 5.0 μ m in size and include organic and inorganic, radioactive and non-radioactive, cytotoxic and inert and those having different degrees of solubility in body fluids.

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(2) To measure the rate of flow and composition (chemical and cellular) of pulmonary (right duct) lymph and thoracic duct lymph before and after the inhalation of various particles.

(3) To determine the number and types of cells in lymph from the right duct (RD) and thoracic duct (TD) after inhalation of various particles. Lymph from the RD is mainly pulmonary and cardiac in origin; lymph from the TD is mainly from the abdominal viscera and lower extremities.

In order to design the experiments the principal investigator, through the kindness of Dr. Ruy Lourenco, spent two half days with Dr. Peter Lee and Mr. Frank J. Hass in Dr. Lourenco's laboratory at the University of Illinois Medical School. A spinning disc generator was in use there to create monodispersed particles for administration to patients for lung scanning. Also, through the cooperation of Dr. R. O. McClellan and the staff of the Inhalation Toxicology Research Institute of the Lovelace Foundation of Albuquerque, New Mexico, three days were spent learning about the administration and quantitation of inhaled particles. The protocol to be presented was discussed with radiobiologists, aerosol physicists, chemists, physiologists, pathologists, etc., to whom we are indebted.

8. Brief statement of working hypothesis.

(1) The alveolar epithelium is relatively and selectively impermeable to many substances, but relatively permeable to others (6).

(2) Protein molecules are rapidly absorbed from the lungs of guinea pigs and dogs and appear in the blood (7,8,9,10,11). The role of the lymphatics in this absorption is not clear. Myer *et al* (12) suggest that ^{131}I -albumin is absorbed from the alveoli more rapidly by pulmonary capillaries than by pulmonary lymphatics. If so, this is different than the absorption of protein molecules from most tissues where the lymphatics are necessary for transfer of molecules from interstitial tissue to the venous circulation (13).

(3) Inhaled particles appear in interstitial tissue and hilar lymph nodes; therefore they must be absorbed by the alveoli and are presumed to be carried by parenchymal lymphatics to the hilar nodes. Inhaled particles may appear within the paraesophageal and paragastric (lesser curvature) lymph nodes of man (14). The para-aortic (abdominal) lymph nodes can receive lymph from the basal lobes of both lungs (15). Lauweryns (16) described juxta-alveolar lymph ducts which are presumed to conduct particles from the alveoli to the interstitial lymphatics. Whether the particles are free or engulfed by macrophages is not the concern of this investigation (17).

(4) Rate of transport of radioactive particles to hilar nodes and blood has been calculated by the use of lung models. The rate of lung node to lung concentration increases with time (1,2,3).

(5) Hard evidence of the presence of particles in pulmonary lymphatics is sparse. Lauweryns (18) demonstrated ferritin and carbon in interstitial lymphatics of the lung after endotracheal instillation. Morrow and Casarett (19)

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have demonstrated particles and particular aggregates in peribronchial lymphatics of dogs exposed to a PuO_2 aerosol. The permeability of lymphatics of the leg to substances of various molecular sizes has been studied by Myerson *et al* (20) by cannulation of afferent and efferent lymphatics of the popliteal node. Garlick and Renkin (21) describe the transport of large molecules from plasma to interstitial fluid and lymph in the paw of dogs in experiments in which lymphatics distal to the popliteal lymph node were cannulated.

(6) Particles may deposit in regional nodes, e.g., hilar nodes or may by-pass them depending on particle size and other less well defined factors (22,23). Deposition of particles can be variable as demonstrated by autoradiograms of adjacent lymph nodes after inhalation of plutonium-239 dioxide. One node contained many particles PuO_2 on the afferent side of the node, and only one or two particles were present in subcapsular sinusoids of the other(19).

(7) It is suggested that examination of pulmonary lymph and pulmonary vein blood after inhalation of particles may demonstrate the presence of particles which have by-passed hilar nodes. We have developed techniques for the collection of pulmonary and systemic lymph (24,25,26). By our improved method of collecting pulmonary lymph, the entire output of the RD is obtained for cellular and chemical examination and for the detection of particles.

(8) The ingestion of aerosol particles by the intestinal tract may be followed by the appearance of particles in thoracic duct lymph and blood. For this reason concomitant collection of TD lymph is necessary.

(9) Alterations in total and differential counts of cells in RD and TD lymph (26) after inhalation of particles are an index of the body's response to the effects of such inhalations. This may give new information on the effects of air pollution on the organism, particularly when particles are injurious.

(10). To summarize: Appearance of inhaled particles in pulmonary lymph may be dependent mainly on size (20). Small particles (less than 1 μm) are probably more likely to by-pass or traverse hilar nodes than larger particles. Solubility, cytotoxicity, chemical structure, i.e. organic or inorganic, electrical charge, etc. are other factors which may influence alveolar absorption and appearance of particles in lymph. By studying a series of particles of various sizes and chemical characteristics information on the role of the lymphatics in the absorption of particles may be obtained.

9. Details of experimental design and procedures.

- (1) Collection of lymph from RD, TD and lobular lymphatic.
- (2) Collection of blood: pulmonary venous (lobular) and peripheral blood.
- (3) Administration of particles: endobronchial instillation, ultrasonic nebulized aerosol, and other methods of aerosol administration.

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- (4) Measurement of concentration of particles in lymph and blood.
- (5) Measurement of particles in hilar lymph nodes by light microscopy, electron microscopy and autoradiographs.
- (6) Experiments in which hilar lymph nodes are excised.
- (7) Cellular composition of lymph.

(1) Collection of lymph. The TD is cannulated in the neck near its junction with the venous system. Cream is given by stomach tube 2 hours prior to cannulation to facilitate visualization of the TD. The chest is not opened for either TD or RD cannulation.

The RD is cannulated in the neck by the improved method described by Leeds *et al* (24) and by Uhley *et al* (25). Evans blue dye (T-1824) is instilled into the right lung in order to aid in visualizing the leash of fine lymphatics which makes up the RD. A segment of the right external jugular and subclavian veins into which the pulmonary lymphatics drain is isolated by dissection and ligation, taking care to preserve the lymphatics. The segment of the external jugular vein is then cannulated and the entire pulmonary lymph drainage, except for a portion from the left upper lobe, is collected. This is an improvement over the method of Warren and Drinker (27) published in 1942, in which one small lymphatic was cannulated and only a small portion of the pulmonary flow obtained. Five hourly samples of lymph from the RD and TD are usually collected for measurement of rate of flow and for cellular and chemical analysis.

(2) Collecting blood. Peripheral venous blood is drawn from the femoral vein. Pulmonary venous blood is drawn from a lobular vein.

(3) Administration of particles. Solutes and suspensions of protein molecules such as ^{131}I -albumin can be administered by passing a 16 gauge catheter through a cuffed endotracheal tube or bronchoscope into the R and L mainstem bronchus and injection of the material with a syringe.

Aerosols containing non-radioactive or radioactive colloids or other particles are administered by an ultrasonic nebulizer. (Mistogen Electronic Nebulizer, Model EN 145, 2711 Adeline St., Oakland, California 94607). With this apparatus approximately 0.5 ml of solution can be converted into a dense aerosol mist per minute. The mean size of the aerosol particles is estimated to be 0.5 to 1.0 μm . A dual valve arrangement maintains flow in one direction in a closed system from nebulizer to subject to vented hood outlet. Radioactive solutions can be used in safety with this apparatus. About 10% of radioaerosol is deposited in the lung (28).

Should the results of experiments with this simple method warrant, a more sophisticated apparatus such as that described by Kanapilly *et al* (29) can be constructed. A spinning disc generator is a useful method to create monodispersed particles (30). Current techniques permit measurement of the size and concentration of particles delivered (31,32).

(4) Measurement of concentration of particles in lymph and blood. Non-radioactive particles are measured by light and electron microscopic methods. Particles can be counted in a hemocytometer in an aliquot of lymph.

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Radioactive particle concentrations in lymph or pulmonary venous blood are measured in a scintillation counting chamber.

The size of particles can be measured with a Porton graticule and stage micrometer (31).

Autoradiography techniques described by Leary (33), Evans (34) and Arnold (35) can be used to identify and measure radioactive particles with light microscopy. Electron microscopy can be used for radioactive and non-radioactive particles after they are collected on membrane filters and transferred to a carbon-coated electron microscope grid (36).

(5) Measurement of particles in hilar lymph nodes. Autoradiography of histologic sections as described by Evans (34), and Arnold (35) permits microscopical determination of the distribution of radioactive materials in the tissues. Particle size analysis is also achieved by autoradiography.

(6) Experiments in which hilar lymph nodes are excised. To determine the role of hilar lymph nodes in the removal of particles absorbed by the pulmonary alveoli, the experiments described above (Section 9 (3)) can be repeated in dogs in which the hilar and mediastinal lymph nodes have been excised. A sternal splitting incision gives access to the lymph nodes of the hilum of each lung, peritracheal and carinal areas. The hilar lymph nodes are excised and a period of 2 weeks or more allowed for regeneration of the pulmonary lymphatic pathways. Particles are administered after cannulation of the RD or lobular lymphatic and measurement of flow and composition of pulmonary lymph. Samples of pulmonary lymph are collected at 1/2 hourly intervals after inhalation of particles and the lymph examined for the presence of particles.

(7) All samples of RD and TD lymph are examined to determine total number of cells and differential counts of RBC and WBC (26).

10. Space and facilities available.

These include a modern experimental surgery laboratory, opened in 1963, with four operating tables each with piped-in suction, air and oxygen, special lights and Bird and Harvard respirators. Large preparation and work rooms adjoin. In addition, there is an individual room for the use of the investigators and technician personnel, which contains benches, desk, record files, book shelves, storage cupboards and drawers for special equipment. Major items of equipment which are available or permanently kept in the laboratory are: 6-channel Electronic for Medicine recording oscillograph, portable x-ray machine, electron microscope, blood gas analysis apparatus, centrifuge, Mistogen electronic nebulizer, fume hood and a Zeiss operating microscope equipped with camera and Strobe light. In addition, the facilities of the Division of Nuclear Medicine are available for determination of radioactivity in blood and lymph and for scintillation scans and radioautographs of the lung. The facilities of the Immunology Research Laboratory are available for cell counts of lymph, hematologic and immunologic (i.e. electrophoresis) studies. The facilities of the Pulmonary Laboratory are available for administration

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of aerosol particles and gases. The personnel of these three sections collaborate closely with the principal investigator and his associates in a team effort. All the above facilities are under one roof, the Mount Zion Hospital and Medical Center.

11. Additional facilities required: None
12. Biographical sketches of investigators and other professional personnel: See Appendix I., pp.12-19.
13. Publications: See Appendix II., p.20.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

| | | |
|--|----|--------|
| Sanford E. Leeds, M.D., Principal Investigator | 40 | 12,000 |
| Philip E. Mann, M.D., Co-Investigator | 10 | 2,500 |
| Herman N. Uhley, M.D. | 5 | 0 |
| Theodore N. Finley, M.D. | 5 | 0 |
| Kenneth R. McCormack, M.D. | 5 | 0 |
| Jay Gershow, M.D. | 5 | 0 |

Technical

| | | |
|-------------------------|-----|--------|
| Technician II (Aerosol) | 100 | 12,000 |
|-------------------------|-----|--------|

| | | |
|---------------------|--|-------|
| Fringe benefits 15% | | 4,194 |
|---------------------|--|-------|

| | |
|-----------------|--------|
| Sub-Total for A | 30,694 |
|-----------------|--------|

B. Consumable supplies (by major categories)

| | |
|---|-------|
| Dogs at \$25.00 each | 2,000 |
| Surgical supplies, endotracheal tubes, plastic tubes, etc. | 500 |
| Radioactive isotopes, chemicals | 1,000 |
| Oxygen, miscellaneous gases | 200 |

| | |
|-----------------|-------|
| Sub-Total for B | 3,700 |
|-----------------|-------|

C. Other expenses (itemize)

| | |
|--|-----|
| Publication | 300 |
| Photography, Medical Artist | 75 |
| Lab. tests, Nuclear Medicine Lab. costs | 500 |
| Travel to meetings on aerosols, air pollution, etc. | 500 |

| | |
|-----------------|-------|
| Sub-Total for C | 1,375 |
|-----------------|-------|

| | |
|----------------------------|--------|
| Running Total of A + B + C | 35,769 |
|----------------------------|--------|

D. Permanent equipment (itemize)

| | |
|--|-------|
| Parts to build aerosol administration apparatus (aerosol generator, nebulizer, heating column, filters, etc., cascade impactor, electrostatic precipitator, etc.) | 3,000 |
|--|-------|

| | |
|-----------------|-------|
| Sub-Total for D | 3,000 |
|-----------------|-------|

E. Indirect costs (15% of A + B + C)

| | |
|---|-------|
| E | 5,365 |
|---|-------|

| | |
|---------------|--------|
| Total request | 44,134 |
|---------------|--------|

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 32,382 | 3,700 | 1,375 | 2,000 | 5,618 | 45,075 |
| Year 3 | 34,163 | 3,700 | 1,375 | 2,000 | 5,865 | 47,123 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|---|--------------------|----------------------------|
| Relation of lymphatics to cardiopulmonary function. | Department of Health, Education and Welfare HL 3180-16 to 3180-20 | 276,000 | Dec.1, 1973 - Nov.30,1977. |
| | | 2nd year 53,093 | Dec.1, 1973 - Nov.30,1974. |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| None | | | |

is understood that the investigator and institutional
officers in applying for a grant have read and accept
the Council's "Statement of Policy Containing Conditions
and Terms Under Which Project Grants Are Made."

Checks payable to
Mount Zion Hospital and Medical Center

Mailing address for checks
Mr. George A. Thompson, Controller
Mount Zion Hospital and Medical Center
P. O. Box 7921, San Francisco, Cal. 94120

Principal investigator

Sanford E. Leeds, M.D.

Typed Name

Signature Sanford E. Leeds Date 1/24/74Telephone 415 567-0950

Area Code

Number

Extension

Responsible officer of institution

Typed Name Jay Okun YedwabTitle Executive DirectorSignature Jay Okun Yedwab Date 1/25/74Telephone 415 567-6600 201

Area Code

Number

Extension

1003540071

REFERENCES

1. Morrow PE: Deposition and retention models for internal dosimetry of the human respiratory tract. Health Phys 12:173-206, 1966.
2. Dyson ED, Beach SA: The movement of inhaled material from the respiratory tract to blood: an analogue investigation of the new lung model. Health Phys 15:385-397, 1968.
3. Thomas RG: Transport of relatively insoluble materials from lung to lymph nodes. Health Phys 14:111-117, 1968.
4. Mercer TT: On the role of particle size in the dissolution of lung burdens. Health Phys 13:1211-1221, 1967.
5. Morrow PE: Lymphatic drainage of the lung in dust clearance. Ann NY Acad Sci 200:46-65, 1972.
6. Taylor AE, Guyton AC, Bishop VS: Permeability of the alveolar membrane to solutes. Circ Res 16:353-362, 1965.
7. Liebow AA: Aspects of transcapillary fluid and protein exchange in the lung. In: The Pulmonary Circulation and Interstitial Space. AP Fishman & HH Hecht, eds., U. Chicago Press, Chicago, Ill., pp.99-112, 1969.
8. Dominguez EAM, Liebow AA, Bensch CG: Studies on the pulmonary air tissue barrier. I. Absorption of albumin by the alveolar wall. Lab Invest 16:905, 1967.
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10. Schultz AL, Grismer JT, Graude F: Absorption of radioactive albumin from the lungs of normal dogs. J Lab Clin Med 61:494-500, 1963.
11. Schultz AL, Grismer JT, Wada S, Graude F: Absorption of albumin from alveoli of perfused dog lung. Am J Physiol 207:1300-1304, 1964.
12. Meyer EC, Dominguez EAM, Bensch KG: Pulmonary lymphatic and blood absorption of albumin from alveoli. A quantitative comparison. Lab Invest 20:1-8, 1969.
13. Yoffey JM, Courtice FC: Lymphatics, Lymph and The Lymphomyeloid Complex. Acad. Press, Inc., New York City, N.Y., 1970.
14. Oliver RS, Watson S: The lymphatic system - a storehouse of longstay deposits of inhaled radioactive particles. Health Phys 12:270, 1966.
15. Meyer KK: Direct lymphatic connections from the lower lobes of the lung to the abdomen. J Thorac Surg 35:726-733, 1958.
16. Lauweryns JM: The juxta-alveolar lymphatics in the human adult lung. Am Rev Respir Dis 102:877-885, 1970.

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17. Brain JD: Free cells in the lungs. Arch Int Med 126:477-487, 1970.
18. Lauweryns JM: The role of the lung lymphatics in the transport of intratracheally instilled particles. 4th Internat Congress of Lymphology, March 1973, Abstract, p.51.
19. Morrow PE, Casarett LJ: An experimental study of the deposition and retention of a plutonium-239 dioxide aerosol. In: Inhaled Particles and Vapours. C.N. Davies, ed., Vol.1:167-175, Pergamon Press, 1961, N.Y.
20. Mayerson HS, Patterson RM, McKee A, LeBrie SJ, Mayerson P: Permeability of lymphatic vessels. Am J Physiol 203:98-106, 1962.
21. Garlick DG, Renkin EM: Transport of large molecules from plasma to interstitial fluid and lymph in dogs. Am J Physiol 219:1595-1605, 1970.
22. Ludwig J: Trapping of calibrated microsphere in rat lymph nodes. Lymphology 4:18-24, 1971.
23. Allen L, Weatherford T: Role of fenestrated basement membrane in lymphatic absorption from peritoneal cavity. Am J Physiol 197:551-554, 1959.
24. Leeds SE, Uhley HN, Sampson JJ, Friedman M: A new method for measurement of lymph flow from the right duct in the dog. Am J Surg 98:211, 1959.
25. Uhley HN, Leeds SE, Sampson JJ, Friedman M: Right duct lymph flow in dogs measured by a new method. Dis Chest 37:532, 1960.
26. Leeds SE, Uhley HN, Basch CM, Rosenbaum EH, Yoffey JM: Comparative study of lymph and lymphocytes of the thoracic and right lymphatic ducts. I. Normal dogs. Lymphology 4:53-57, 1971.
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30. Lippmann M, Albert RE: A compact electric motor driven spinning disc aerosol generator. Am Indust Hyg Assn 28:501, 1967.
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32. Raabe OG: Instruments & Methods for Characterizing Radioactive Aerosols. IEEE Transactions on Nuclear Science Vol. NS-19, No.1, pp.64-75, Feb. 1972.

1003540073

References, continued:

33. Leary JA. Particle size determination in radioactive aerosols by radioautograph. Anal Chem 23:850, 1951.
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1003540074

#948 - LENTZ

1003540075

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

November 29, 1973

Grant application No. 948

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Gardner, Liebow, and Sommers

Subject: Patrick E. Lentz, Ph.D., Tulane Univ. School of Medicine, New Orleans
New application No. 948
"Cigarette Smoke and Transport of Substrates by Alveolar Macrophages"

History

This proposal was case No. 235, and application was encouraged.

Request

Application No. 948 requests \$20,626.00 plus two additional years.

Documents Submitted (attached)

1. Application, undated, received by CTR November 19, 1973.
2. Manuscripts of the three papers checked on page 3E of the application.

FWN:gh
Enclosures


F.W.N.

1003540076

Committee:
Dr. Gardner
Dr. Liebow
Dr. Sommers

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

NOV 19 1973

Application for Research Grant
(Use extra pages as needed)

NOV 19 1973

Date:

1. Principal Investigator (give title and degrees):

Patrick E. Lentz, Ph.D.

2. Institution & address:

Tulane University School of Medicine
1430 Tulane Avenue
New Orleans, Louisiana 70112

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

Cigarette Smoke and Transport of Substrates by Alveolar Macrophages

5. Proposed starting date: January, 1974

6. Estimated time to complete: 36 months

7. Brief description of specific research aims:

Recent studies in this laboratory have demonstrated that aqueous extracts of cigarette smoke inhibit the ability of alveolar macrophages to actively transport the non-metabolizable amino acid, α amino isobutyric ^{14}C - (AIB). The aim of this research program is to examine the mechanisms whereby certain components of cigarette smoke interfere with the transport of AIB across the membrane of alveolar macrophages.

The specific objectives of this investigation are:

- 1) to establish the dose response and time course relationships for the effects of aqueous extracts of cigarette smoke (AECS), nicotine, and cadmium on the active accumulation of a non-metabolizable amino acid, AIB, by alveolar macrophages
- 2) to determine the effects of AECS, nicotine, and cadmium on the kinetic parameters of active accumulation of AIB
- 3) to study the relationship between the effects of AECS, nicotine, and cadmium on AIB transport and the concentration of ATP in alveolar macrophages
- 4) to study the relationship between the effects of AECS, nicotine or cadmium on AIB transport, and alteration of the nature or active site of the carrier.

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8. Brief statement of working hypothesis:

2.

Alveolar macrophages are a vital component of the pulmonary host defense systems. These cells are responsible for phagocytosis, killing and degradation of bacteria which penetrate to the alveolar surface. Since the functional integrity of the alveolar macrophage depends, to a certain extent, on the availability and membrane transport of essential substrates, our observations of a depression in active transport after exposure to extracts of cigarette smoke may have important clinical significance in pulmonary disease states associated with inhalation of cigarette smoke.

I have formed the working hypothesis that certain components of cigarette smoke impair the ability of alveolar macrophages to actively transport an amino acid either by decreasing the quantity of binding sites or carrier molecules in the cell membrane or by reducing the energy supply available for transport.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached pages.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Adequate laboratory and office space are provided by the Department of Physiology. Facilities for purchase and maintenance of rabbits are available in the Department of Vivarial Science, Tulane University School of Medicine.

Major items of equipment available in this Department include: one Filtrona Smoking Machine, one PR-6 Refrigerated Centrifuge, one Nuclear Chicago Liquid Scintillation Counter, one Beckman DBG Spectrophotometer, microscopes, vacuum oven.

11. Additional facilities required:

An additional Dubnoff shaking water bath is requested.

1003540079

12. Biographical sketches of investigator(s) and other professional personnel (append):

CV append.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Append list and reprints.

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PATRICK EDMUND LENTZ, Ph.D.

BIRTHDATE:

PLACE: ~~REDACTED~~

Married

EDUCATION:

| Institution | Date | Degree |
|---|------|--|
| Loras College Dubuque, Iowa | | Bachelor of Arts, Biology |
| Marquette University Milwaukee, Wisconsin | | Master of Science, Medical Physiology |
| Marquette University Milwaukee, Wisconsin | | Completion Academic Requirements for Doctor of Philosophy |
| University of Wisconsin Madison, Wisconsin | | Special Student (Doctoral research with Dr. J. L. Van Lancker) |
| Brown University Providence, R.I. | | Special Student |
| Marquette University Milwaukee, Wisconsin | | Doctor of Philosophy Degree in Medical Physiology |

POSITIONS:

| Institution | Date | Position |
|---|-----------|--|
| Brown University Providence, R.I. | 1967-1968 | Research Associate, Division of Biological and Medical Science |
| Tulane University School of Medicine, New Orleans | 1969- | Assistant Professor, Department of Physiology |

FELLOWSHIPS:

1. Predoctoral Trainee, United States Public Health Service, 1962-1966.
2. Predoctoral Fellowship, United States Public Health Service, 1966-1967.

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PROFESSIONAL SOCIETIES:

REDACTED

RESEARCH EXPERIENCE:

- 1962-1964 Research on the role of the functional status of the reticuloendothelial system in the pathogenesis of hemorrhagic shock.
- 1964-1967 Research on the site of synthesis of beta glucuronidase in regenerating rat liver.
- (a) studies on the site of synthesis and transfer of beta glucuronidase from the microsomal to the small mitochondrial or lysosomal fraction.
- (b) comparison of the catalytic properties of beta glucuronidase purified from microsomal and small mitochondrial fractions.
- 1967-1968 1. Research on the localization, mode of attachment and release of beta glucuronidase from microsomal sub-fractions.
2. Research on the site of synthesis of glutamic dehydrogenase.
- 1969-1970 1. Biochemical characterization of parenchymal and Kupffer cells isolated from rat liver.
- 1971-present 1. Synthesis of RNA, DNA and protein by parenchymal and Kupffer cells isolated from rat liver.
2. Effect of actinomycin D, chloromphenicol and puromycin on nucleic acid and protein synthesis in parenchymal and Kupffer cells isolated from rat liver.
3. Specificity and immunogenicity of RNA synthesized in macrophages after exposure to antigens, such as sheep erythrocytes, E. coli and BP-4 tumor cells.

1003540081

7c

4. Metabolic, phagocytic and bactericidal activities of alveolar macrophages exposed to air pollutants such as cigarette smoke.

RESEARCH INTEREST:

1. Physiological role of parenchymal and Kupffer cells in liver metabolism.
2. Effects of nutritional status, and drugs on metabolism of liver Kupffer and parenchymal cells.
3. Effects of air contaminants (cigarette smoke, oxides of nitrogen and sulfur) on metabolic and bactericidal activities of pulmonary alveolar macrophages.
4. Molecular mechanisms of the immune response. Role of macrophage RNA in the initiation of an immune response to specific antigens.

1003540082

34

PUBLICATIONS:

- Lentz, Patrick E., James M. Lipo and William J. Stekiel. Effect of hemorrhagic shock on rat plasma lysosomal hydrolase activity. Fed. Proc. 24, 1965 (Abstract).
- Lentz, Patrick E. and James J. Smith. The effect of reticuloendothelial stimulation on plasma hydrolase activity in hemorrhagic shock. Proc. Soc. Exp. Biol. Med. 124:1243-1248, 1967.
- Lentz, Patrick E. Studies on the Site of Synthesis of Beta glucuronidase. Ph.D. Thesis, Marquette University, 1967.
- Lentz, Patrick E. and J. L. Van Lancker. The transfer of beta glucuronidase from the microsomal to the small mitochondrial fraction in hypoxic livers. Am. J. Pathol. 1968 (Abstract).
- Van Lancker, J. L. and Patrick E. Lentz. Molecular mechanisms of liver regeneration. VI. The site of synthesis of beta glucuronidase. J. Histochemistry and Cytochemistry. 18:529-541, 1970.
- Van Lancker, J. L., V. Melnick and P. E. Lentz. Site of synthesis of glutamic dehydrogenase in regenerating rat liver. (manuscript in preparation).
- Lentz, Patrick E. and N. R. Di Luzio. Biochemical characteristics of parenchymal and Kupffer cells isolated from rat liver. Exp. Cell Res. 67:17-26, 1971.
- Lentz, P. E. and N. R. Di Luzio. Isolation of immunogenic RNA from liver, splenic and peritoneal macrophages (accepted RES - J. Reticuloendothelial Soc., 1973).
- Lentz, P. E. and N. R. Di Luzio. Effect of AET (B-amino ethyl isothiourea) and isologous bone marrow on reticuloendothelial function after cobalt-60 irradiation (submitted for publication Radiation Research).
- Lentz, P. E. and N. R. Di Luzio. Biochemical composition of Kupffer and parenchymal cells isolated from normal, RE-stimulated or depressed rats. RES, J. Reticuloendothelial Society 9:609, 1971 (Abstract).
- Lentz, P. E. Isolation of immunogenic RNA from rat macrophages. Abstract of paper presented at Fall Meeting American Physiology Society, August, 1971.

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3e

Lentz, P. E. Bactericidal and metabolic activities of alveolar macrophages exposed to tobacco smoke. Abstract of paper presented at Eighth National Meeting of the Reticuloendothelial Society, Detroit, Michigan, November 30, 1971.

Lentz, P. E. and N. R. Di Luzio. Comparative influence of aqueous extracts of cigarette smoke on phagocytic, bactericidal and metabolic activities of macrophages. Abstract of paper presented at symposium sponsored by American Medical Association Education and Research Foundation, May, 1972.

Pisano, J. C., J. T. Patterson, R. Trejo, E. Hoffmann, P. E. Lentz and N. R. Di Luzio. Hepatotoxic effects of horse anti-mouse lymphocyte serum. Exp. Molecular Pathology 16:302, 1972.

✓ Lentz, P. E. and N. R. Di Luzio. Phagocytic and Bactericidal Activities of rat alveolar macrophages exposed to aqueous extracts of cigarette smoke in vitro. (submitted Am. Rev. Resp. Disease).

Lentz, P. E. Effects of actinomycin D, puromycin and chloramphenicol on nucleic acid and protein synthesis by Kupffer and parenchymal cells isolated from rat liver. Rush Presbyterian St. Luke's Medical Bulletin 12:211-221, 1973.

(Lentz, P. E. and N. R. Di Luzio. Isolation of adult rat liver macrophages (von Kupffer cells). in Biomembranes (Cells, Organelles and Membraneous Components). A volume of Methods in Enzymology. ed. S. P. Colowick and N. O. Kaplan. Academic Press, Inc. New York, NY, Accepted for publication.

Lentz, P. E. and N. R. Di Luzio. Functional alterations in alveolar macrophages exposed to cigarette smoke in vitro and in vivo. Abstract of paper to be presented at 9th National Meeting of the Reticuloendothelial Society. December, 1972.

✓ Lentz, P. E. and N. R. Di Luzio. Transport of alpha aminoisobutyric acid by alveolar macrophages incubated with cigarette smoke and nicotine. (Submitted Arch. Environ. Health, 1973).

✓ Lentz, P. E. and N. R. Di Luzio. Peroxidation of lipids in alveolar macrophages and pulmonary protective factor by aqueous extracts of cigarette smoke (submitted Arch. Environ. Health, 1973).

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14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)Patrick E. Lentz, Ph.D.
Principal Investigator
Fringe Benefits

22

REDACTED

Technical

Research Technician, Grade III
Fringe Benefits

100

REDACTED

REDACTED
REDACTED

Sub-Total for A

B. Consumable supplies (by major categories)

Animals 160 rabbits @ \$10 ea.
Isotopes α -aminoisobutyric acid -1- ^{14}C
inulin carboxy ^{14}C

1,600.00

600.00

Scintillation counting supplies
Laboratory chemicals and glassware
Cigarettes

800.00

600.00

100.00

Sub-Total for B

3,700.00

C. Other expenses (itemize)

Travel one National Meeting

300.00

Sub-Total for C

300.00

Running Total of A + B + C

17,588.00

D. Permanent equipment (itemize)

1 Dubnoff Shaking Water Bath

400.00

Sub-Total for D

400.00

E. Indirect costs (15% of A+B+C)

E

2,638.00

Total request

20,626.00

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|-------------|
| Year 2 | REDACTED | 3,700.00 | 300.00 | -0- | 2,753.00 | \$21,104.00 |
| Year 3 | | 3,700.00 | 300.00 | -0- | 2,886.00 | \$22,126.00 |

1003540085

5.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

| CURRENTLY ACTIVE | | | |
|------------------|--------------------------------|--------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| None | | | |

| PENDING OR PLANNED | | | |
|---|--------------------------------------|---------|----------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| Transport of Glucose By Alveolar Macrophages | National Heart and Lung Institute | 30,000. | 2 years July 1974-76 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Principal investigator

Typed Name Patrick E. Lentz, Ph.D.Signature *Patrick E. Lentz* Date 11/7/73Telephone Area Code Number Extension

Checks payable to

(Tulane University

Mailing address for checks

Jesse B. MorganBusiness Manager and ComptrollerTulane University
New Orleans, La.

Responsible officer of institution

Typed Name J. T. Hamlin, III, M.D.Title Vice-DeanSignature *J. T. Hamlin, III* Date 11/7/73Telephone Area Code Number Extension

1003540086

I. Amino Acid Transport in Mammalian Cells

The cell membrane has the capacity to transport solutes into and out of the cell. These transport processes are responsible for maintaining the constancy of the internal environment of the cell and providing the substrates required for cellular metabolism, and synthetic activities. Within the last decade the molecular mechanisms underlying membrane transport have begun to be unraveled, and certain characteristics of the transport process are now well established. Kinetic studies with bacterial and mammalian cells have provided convincing evidence for mobile membrane carrier systems with variable degrees of specificity for a variety of solutes. Unfortunately, the chemical nature of the carrier has not been established, and until such data are available, there will be a considerable gap in our understanding of the precise mechanism of operation of the transport system.

This review of the literature will be restricted to: 1) the active transport of amino acids in animal cells; the many interesting features of inorganic ion, water, and carbohydrate transport in both mammalian and bacterial cells has been reviewed recently (1, 2, 3, 4, 5); and 2) the influence of air contaminants on membrane function.

A. Terminology

1. Active transport is defined as the net movement of a solute against an electrochemical gradient with expenditure of metabolic energy. From an operational point of view, an active transport system carries out a three step process: a) binding of a solute to a receptor site; b) translocation of the solute-receptor complex across the plasma membrane; and c) coupling of the process to metabolic energy.

2. Facilitated diffusion is a carrier mediated process of solute uptake which does not require utilization of metabolic energy under normal physiological conditions.

3. Transport system, carrier, membrane carrier are used interchangeably to identify a system, obeying saturation kinetics, which facilitates movement of specific solutes across cytoplasmic membranes. Also signifies the part of the transport system which determines its specificity.

4. Distribution Ratio is defined as the ratio of the concentration of solute in the intracellular water to that in the extracellular water, if and only if the solute is free in the aqueous phase and not metabolizable by the cell.

5. Steady state signifies the maximum intracellular concentration of the solute which can be attained by the cell under defined conditions of pH, solute concentration, and temperature.

B. Amino Acid Transport in Animal Cells

The first observations pointing to a "concentrative uptake" by animal cells were probably made by Van Slyke and Meyer, who

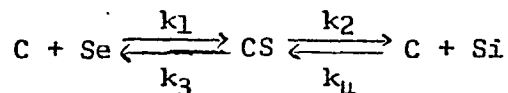
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in 1913 found that amino acids injected intravenously rapidly disappeared from blood (6). The important pioneering work in studying accumulation of amino acids by animal cells was carried out by Christensen and colleagues (7, 8, 9, 10, 11) who tested several types of cells for their ability to concentrate each amino acid out of the whole spectrum of natural amino acids and some of their close analogs. Since Ehrlich ascites carcinoma cells (Ehrlich cells) concentrate all the natural amino acids, and maintain higher distribution ratios than most other cells (12), they have been more extensively employed for studies on amino acid transport than other cells. Although detailed studies have been made of amino acid transport in intestine, kidney, and erythrocytes, the general conception of membrane transport of amino acids is derived from its characterization in the Ehrlich cell (13). Data from other types of cells support the concept that most, if not all, living cells are able to transport at least some amino acids, although different cellular species may differ greatly with respect to the development of particular amino acid transporting systems (2, 4, 5).

B.1. Kinetics of Amino Acid Transport

Transport of most natural amino acids, and analogs of amino acids, appears to be mediated by a membrane carrier that binds the substrate before translocating it from the outer to the inner surface of the plasma membrane. This model is inferred from criteria, such as saturation kinetics, specificity, and competitive inhibition by other amino acids, that are considered to be characteristic of carrier mediated transport (14).

The transport of natural amino acids shows saturation kinetics that often fit the Michaelis Menten relationship, and double reciprocal plots of initial velocity against substrate concentration are linear, at least in the lower ranges of concentration. Since the kinetics of carrier mediated transport are similar to enzyme kinetics, the initial reactions of transport are often represented by the simplified model (15, 16, 17):



where C represents the carrier, and Se, and Si represent the extra- and intra- cellular concentration of substrate, respectively. CS represents the carrier substrate complex and k (k_1 , k_2 , k_3 , k_4) represents the rate constants.

If the total number of carriers is represented by Ct, the maximal rate of unidirectional influx can be expressed by equation 1:

$$V_{max} = k_2 (CtS) \quad (1)$$

Under defined conditions, V_{max} is directly proportional to the number of membrane carriers. The initial rate of transport (V_0),

at a single concentration of substrate, is determined by equation 2:

$$V_o = V_{max} \left(\frac{(S)}{(S) + K_m} \right) \quad (2)$$

where K_m is the substrate concentration which gives half maximal velocity. Thus, the initial rate of transport at any single concentration of substrate is determined by the following relationship:

$$V_o = k_2 (CtS) \left(\frac{(S)}{(S) + K_m} \right) \quad (3)$$

From equation 3 it is apparent that the initial rate of influx transport is influenced by three factors: k_2 , (CtS) , and K_m . It is assumed in this discussion that k_2 can be influenced only by exchange diffusion (stimulation of solute flux by the intracellular concentration of solute). The number of membrane carriers are reflected by the initial velocity under different experimental conditions only when k_2 and K_m remain constant.

The kinetic equations described above apply to the carrier mediated flux of substrates from the extracellular to the intracellular water. These equations do not include the contribution of simple passive diffusion which occurs when $S_e > S_i$.

Christensen and Handlogten (18) have reached the conclusion that amino acids escape from the Ehrlich cell via a carrier mediated route similar to that used for uptake. In addition as the concentration of S_i increases back diffusion will increase. Thus a set of equations similar to those derived above can be written for the unidirectional flux of substrate from the intracellular water to the extracellular water. Relatively little information is available on the characteristics of carrier mediated efflux. Christensen and Handlogten (18) have demonstrated that the K_m values for carrier mediated efflux were, in general, much larger than those for influx, whereas, the V_{max} values for the two directions were similar.

These results imply that the "apparent affinity" of the carrier for efflux is much lower than for influx and that under the normal conditions of transport studies (ie., short incubation times) the amount of substrate pumped from the cells may be small.

Therefore the net flux of substrate across the cell membrane is described by:

$$M_s^{e \rightarrow i_{net}} = M_s^{e \rightarrow i} - M_s^{i \rightarrow e}$$

where $M_s^{e \rightarrow i}$, and $M_s^{i \rightarrow e}$ represent the movement of substrate from extracellular (e) to intracellular (i), or vice versa.

Each of these unidirectional fluxes is composed of a carrier mediated and passive diffusion component, and the steady state condition will be given by:

$$M^{e \rightarrow i} \text{ steady state} = \left[k_2 C_t S \frac{(Se)}{(Se) + K_m} + k_D (Se - Si) \right] - \left[k_4 C_t S \frac{Si}{Si + K_m} + k_D (Si - Se) \right]$$

where k_D is the diffusion constant of the substrate.

Even in cases of a straight and homogenous Michaelis-Menten relationship the interpretation of K_m and V_{max} is difficult (14). K_m , the half saturation constant, is not an accurate index of the affinity between the carrier and amino acid because K_m and V_{max} are dependent on the mobility of the loaded and unloaded carrier. If the loaded and unloaded carrier have different rate constants, the initial velocity of transport would be determined by the respective concentrations of loaded and unloaded carrier at any given time. Thus, any factor which influences the ratio of these two rate constants would change K_m even though the affinity of the carrier for the substrate is unchanged. In a similar manner, V_{max} , which is often interpreted as the product of the total number of carriers, and the rate coefficient for the loaded carrier, may be limited more by the supply of energy (ATP) than by these two factors.

In spite of these reservations about the interpretations of V_o , V_{max} , and K_m , determinations of these parameters of transport have provided valuable insights into the mechanisms by which hormones (1, 19, 20) and drugs (1) influence transport of amino acids in cells and tissues. These terms will be defined and interpreted in this research project as follows:

- 1) Initial velocity (V_o): rate of transport at a single concentration of substrate; reflection of the total number of carriers if k_2 and K_m are unchanged.
- 2) Maximum velocity (V_{max}): total capacity of the system; reflection of the amount of energy available to the transport system.
- 3) Michaelis-Menten constant (K_m): concentration of substrate which yields half maximal velocity; reflection of "apparent affinity" or "nature" of the carrier.

B.2. Specificities and Special Systems for Transport of Amino Acids

Transport systems, like enzymes, are specific for certain amino acids or groups of amino acids. As a general rule, transport systems are less specific than enzymes, since they only prefer certain amino acids, and there is considerable overlapping specificity between the different systems (1, 21). For example: L-amino acids are transported more rapidly than the D-configuration; neutral amino acids require a free carboxyl and free amino group in the alpha position and a nonpolar side chain; replacement

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of the nonpolar group with anionic or cationic groups will abolish the affinity of the amino acid for neutral transport systems, and increase the affinity for a system specific for acidic or basic amino acids.

The most extensive characterization of the specificities of different systems for amino acid transport has been made by Oxender and Christensen (22). Their studies have identified several systems for transport of neutral, basic and acidic amino acids. The characteristics of the "A" system are included in this discussion because alpha amino isobutyric acid (AIB), the amino acid analog used in this research program, is known to be transported by this system.

A system (13)

defined in: Ehrlich Cell

Substrates: all neutral amino acids (alanine, glycine, proline) and analogs of amino acids, amino isobutyric (AIB) and N-methyl amino isobutyric acid (MeAIB)

V_{max}: constant

dependency on Na ion: first order; causes one sodium to migrate inward per amino acid molecule

pH sensitivity: barely detectable at pH 5

mechanism: active transport

other apparent sources: Hamster intestine, kidney, brain, hepatoma tissue culture cells; conspicuously absent from mature erythrocytes

Although these kinetic studies have characterized several transport systems in Ehrlich cells for neutral, basic and acidic amino acids the degree to which these systems are represented in normal animal cells remains to be established. Attempts have been made to generalize the transport systems of Ehrlich ascites tumor cells to other mammalian cells. However, several differences in the transport systems for lysine in alveolar macrophages and tumor cells are readily apparent (Section C). The extensive studies of Christensen have provided insights into the transport mechanisms, and offer an invaluable guide for studying transport systems in other types of cells.

B.3. Ions and Amino Acid Transport Systems

One of the most interesting aspects of amino acid transport systems in animal cells is a requirement for extracellular sodium. This sodium dependency is characterized by two distinctive features: 1) if sodium ions are removed from the incubation media the uni-directional influx of the amino acid is decreased, and 2) the steady state concentration of the amino acid in the cell is equal to that in the incubation media. These effects may result from a decrease in maximal flux, an increase in apparent Michaelis-Menten constant (decreased affinity), or both, depending on the type of cell and the amino acid.

The effect of alkali metal ions on the transport of amino acids and sugars has been studied in a variety of cells and tissues (1, 3, 4, 10). In most cells, the data support the hypo-

thesis that sodium ion is a co-substrate for the carrier system. That is, movements of sodium ions down their electrochemical gradient provides the ultimate driving force for active accumulation of amino acids. The alveolar macrophage is an exception, and the anomalous effects of Na^+ in this cell are discussed later. There are at least three ways in which sodium ion could act as a co-factor for amino acid transport: 1) facilitation of energy utilization perhaps via Na -dependent adenosine triphosphatase (experimentally measured as an increased V_{max}); 2) increasing the affinity of the carrier for the substrate (experimentally measured as a decreased K_m); or 3) combination of 1&2.

B.4. Energetics of Transport

Active accumulation of amino acids is an energy dependent process. Inhibitors of oxidative metabolism, such as cyanide, dinitrophenol, or anaerobiosis, inhibit accumulation of amino acids in a variety of cells (1, 23). Usually, these experimental conditions do not completely depress the uptake suggesting, perhaps, that anaerobic glycolysis or other sources of energy are available for active transport. As discussed in Section B.3. the sodium gradient hypothesis, rather than direct coupling of energy to transport, appears to provide the proximate driving force for the intracellular accumulation of amino acids. However, in either case of direct or indirect coupling of metabolic energy to amino acid transport, oxidative metabolism must generate ATP, and it is probable that active transport of amino acids is coupled to both ion gradients and expenditure of metabolic energy. Additional studies concerned with the proportion of ATP utilized for operation of the " Na^+ pump" or amino acid transport in a variety of cells are clearly required.

B.5. Chemical Basis for Transport

Most models for transport of amino acids assume that the first step is a specific binding of substrate to a receptor site on a carrier molecule. This binding reaction is then followed by movement of the substrate-carrier complex through the plasma membrane and subsequent release of the substrate inside of the cell. The chemical nature of the carrier molecule remains speculative. In bacterial systems a number of proteins have been proposed as the active carriers involved in transport systems for a variety of amino acids, sugars and ions (5). Unfortunately, similar studies with animal cells has not yet progressed to the same extent.

C.1. Amino Acid Transport in Alveolar Macrophages

Transport of natural amino acids by rabbit alveolar macrophages has been studied extensively by Berlin and colleagues (21). Their studies which used lysine, a dibasic amino acid, and leucine, a neutral amino acid, clearly supported the general conception of a carrier mediated system. However, there are a number of striking differences between the transport systems in alveolar macrophages and Ehrlich cells (24). First, lysine was transported by a single system which had some affinity for all natural amino

acids. In contrast, Ehrlich cells have more than one transport system for dibasic amino acids and have no affinity for acidic or neutral amino acids. Second, neutral amino acids, such as leucine or histidine, completely inhibited lysine transport. This observation implied striking differences in specificity between this cell and epithelial tissues where neutral amino acids do not block transport of dibasic amino acids. Third, leucine was transported by at least two systems: one common with lysine, and the other independent of the lysine. Fourth, there was an inverse relationship between sodium and/or potassium and lysine accumulation. Replacement of sodium with potassium, or choline chloride had no inhibitory effect on lysine transport; substitution of sucrose or mannitol for Na^+ caused a 50% stimulation of lysine transport. In Ehrlich cells, reduction in extracellular Na^+ dramatically reduces the accumulation of all amino acids. These anomalous effects of sodium and potassium were associated with a decreased maximal velocity and were not related to changes in apparent affinity of the carrier for the substrate.

As discussed above the influence of sodium ion on active transport of amino acids by animal cells has been largely explained by the effect of Na^+ to increase the affinity of nonelectrolytes to the carrier or to increase the rate of translocation of Na^+ - substrate - carrier complexes across the membrane, or both (3). In addition, the bulk of the evidence supports the concept that the asymmetry of Na^+ ion distribution provides the ultimate driving force for amino acid accumulation. The accumulation of lysine by alveolar macrophages cannot be explained by the sodium gradient hypothesis because the effects of sodium and potassium are equivalent. The physiological significance of these novel cation effects is uncertain and additional studies with other amino acids are required to identify and characterize the systems for transport of amino acids by cells in the lung.

Carrier mediated transport and phagocytosis are two distinctive mechanisms for transporting substances across plasma membranes of macrophages. Data from a recent study of Tsan and Berlin (17) suggested that the membrane sites for phagocytosis and amino acid transport are topographically distinct. In these studies, alveolar macrophages phagocytized polyvinyl toluene (PVT) spheres before measurement of lysine transport. If the transport and phagocytic sites were intermixed a depression in transport should have been observed because large amounts of membrane (more than 50%) are known to be internalized during phagocytosis. However, there was no difference in transport between phagocytically active and resting cells. These results suggested that 1) the two sites were geographically separate, or 2) carrier molecules were rapidly synthesized and inserted into the membrane of the alveolar macrophage.

These possibilities were investigated by treatment of the alveolar macrophages with p-chloromercuribenzenesulfonic acid (PCMBSA). This agent is a powerful sulfhydryl inhibitor (25) that blocks lysine transport (17), but does not kill or penetrate the membrane of macrophages (17). Tsan and Berlin treated alveolar macrophages with PCMBSA before incubation with PVT and measurement of lysine transport. If new carriers were introduced during phagocytosis, the inhibition of lysine transport should have been at

least partially reversed. However, the depression in lysine transport was identical in control and phagocytically active cells indicating that new carriers were not introduced into the membrane of macrophages during phagocytosis.

The impermeability of membranes to PCMBSA makes this compound an ideal tool to quantitate the number of surface sulfhydryl groups (25). Since the inhibition of lysine transport by PCMBSA was prevented by high concentration of substrate it was probable that this compound reacted with -SH groups in the active site of the lysine carrier. Using radiolabeled PCMBSA, the number of membrane -SH groups reacting with the sulfhydryl agent was measured in the presence and absence of high concentrations of lysine. In these experiments, about 13% of the total number of -SH groups in the membrane were responsible for lysine transport. These studies imply that -SH groups in the macrophage membrane are involved in the transport system for lysine. Unfortunately, similar experiments with other amino acids have not yet been performed and it is not possible to state whether active sites of carriers for other amino acids also require sulfhydryl groups. The importance of these studies is that they clearly demonstrate that transport sites, at least for lysine in the alveolar macrophages are topographically distinct from phagocytic sites and that the active site of the lysine carrier contains -SH groups.

Ukena and Berlin (26) have recently examined the role of the microtubule system in maintaining the topographical distribution of lysine carriers in membranes of the polymorphonuclear leukocyte (PMN). Incubation of PMN with colchicine or vinblastine, plant alkaloids which specifically bind to microtubular proteins of mammalian cells and disrupt microtubular function, had little effect on lysine transport. However, when PMN were allowed to phagocytize inert particles in the presence of these drugs, membrane transport of lysine was markedly decreased. These studies suggested that microtubules may be involved in maintaining the topographical distribution of carriers in cellular membranes, or that the alkaloids destroyed the cells.

The relationship between microtubules, active transport and phagocytosis in alveolar macrophages exposed to air contaminants has never been examined. Studies on this relationship would be attractive with cigarette smoke as the contaminant because nicotine is a major component in smoke (27). Although data are not available, it is possible that nicotine, a plant alkaloid, destroys microtubules, and, in a phagocytizing cell, a depression in transport could occur because of the lack of topographical separation of transport and phagocytic sites.

Recent experiments of Berlin have examined the temperature dependence of lysine and adenosine transport systems in alveolar macrophages (28). These experiments revealed a sharp transition temperature in Arrhenius plots of velocity versus the reciprocal of temperature. In bacterial systems, transition temperatures are characteristic of the fatty acids incorporated into the membrane. Sharp transition points are not usually observed in animal cells because cholesterol usually broadens or masks the transition. Although the precise explanation for these transition points is

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not certain, they may reflect a transition of long chain fatty acids from a tight, crystalline array to a disordered, more liquid, state. An alternative explanation for transition temperatures in transport systems is that the carrier (protein) exists as an equilibrium between conformers which are favored by high or low temperature, respectively. Conformational changes have also been invoked to account for the transition temperature observed with the Na⁺, K⁺ dependent ATP-ase system in membranes (29).

Whether the temperature characteristics of transport are related to phase transitions of lipids or to shifts in the ratio of carrier conformers remains to be seen. However, each mechanism could provide a basis for regulating transport without synthesis or degradation of carrier proteins (28).

II. Alteration of Plasma Membrane Functions by Air Contaminants

Several recent studies have convincingly demonstrated that air contaminants impair the functional properties of alveolar macrophage membranes, as measured by the loss of "adhesiveness," increased uptake of trypan blue, decreased phagocytosis and killing of bacteria, inhibition of membrane bound enzyme activities and impairment of active transport.

A. Permeability Phenomena

Exclusion of vital dyes has been widely used for rapid determination of cellular viability (30, 31). Several investigators have shown that an increased uptake of ionic dyes (trypan blue or eosin y) correlated well with the inability of cells to metabolize substrates or to multiply in tissue culture (32, 33, 34). An increased dye uptake is commonly interpreted as a loss of semipermeability of the membrane and cells that take up dye are referred to as 'dead' cells (30). Air contaminants, such as cigarette smoke (35, 36), aqueous extracts of smoke (37, 38), specific chemicals in smoke (39, 40), ozone (40, 41) or sulfur dioxide (40) have been reported to reduce the ability of cells to exclude trypan blue. In similar studies, I have found the behaviour of alveolar macrophages, incubated in vitro with aqueous extracts of smoke, was equivocal towards trypan blue; some cells contained dye in the nucleus, in the cytoplasm and some in both nucleus and cytoplasm. In addition, in the degree of staining there was variability ranging from light to intense blue. There are a number of objections to dye exclusion as a technique for estimating cellular viability. First, these dyes are normally phagocytized or pinocytized by macrophages (42) and uptake of dye by these cells could be interpreted as a stimulation of membrane activity rather than damage to the membrane. Secondly, this test differentiates damaged from undamaged cells only if the limits of the test are established for the type of cell and the suspending medium being used (30, 43). Since most published reports on dye exclusion properties of cells do not indicate the dye concentration, serum concentration, incubation time or cell concentration, it is difficult to compare the data. Without prior standardization, the data obtained with dye

exclusion test are often erratic and difficult to objectively interpret.

Additional qualitative evidence for alterations in the membranes of cells exposed in vitro to environmental contaminants is the observation that cells develop blebs or blister-like formations, visible with the light microscope (39, 41), or detach from the surface of culture vessels (38, 39, 41). Quantitative evidence for loss of membrane integrity is found in the studies of Hurst and Coffin (44) which demonstrated that exposure of macrophages to 2 ppm ozone for 3 hours enhanced the leakage of lysosomal hydrolases and detachment of cells from culture flasks. Addition of glutathione or cysteine to the incubation media eliminated the loss of cell bound enzyme activity, and Hurst and Coffin suggest that -SH groups may be importantly involved in the maintenance of membrane integrity.

These qualitative and quantitative studies suggest that plasma membranes were severely damaged or altered in order for proteases to leak from the cell, or for the cells to detach. If membrane damage is severe enough cells would lyse, and release a broad spectrum of enzymes into the media or onto the surface of the lung. Holt and Keast have recently reported that a single acute exposure of mice to high concentrations of smoke resulted in an immediate and dramatic loss of macrophages in the lung (35). If these data represent lysis of cells, and not an emmigration of cells from the lung, an observation of considerable clinical importance has been made because intratracheal injections of homogenates of leucocytes or alveolar macrophages can induce emphysema-like lesions in the lungs of dogs (45). Therefore a relationship between cigarette smoke induced death of macrophages, with concomitant release of proteolytic enzymes, and development of emphysema in the smokers' lung may be possible. However, the implications of the studies of Holt and Keast are tempered, somewhat, by the studies of Harris et al. which demonstrated a highly significant increase in the number of macrophages in the lungs of human smokers (46).

B. Phagocytosis, Killing and Pinocytosis

Specific functions of alveolar macrophage membranes have also been reported to be influenced by cigarette smoke. Stillman first observed that a heavy dose of cigarette smoke inhibited the clearance of bacteria in mice (49). Since bacterial clearance is primarily a function of the phagocytic activity of alveolar macrophages, Green and colleagues (39, 48), and Lentz and Di Luzio (49) have studied the influence of aqueous extracts of cigarette smoke, or its gas phase, on the in vitro phagocytic activity of rabbit and rat alveolar macrophages. Both groups observed a marked depression in the uptake and killing of bacteria when aqueous extracts or gas phase smoke were added to the incubation media. Interestingly, the toxic effects of smoke could be reduced, in part, by addition of biological antioxidants, such as glutathione or cysteine, to the incubation media. These studies implied that cigarette smoke oxidized reducing substances in the cells and that these reducing substances (i.e. sulfhydryls) were required for phagocytosis, and the activity of intracellular enzymes responsible

for generation of energy for phagocytosis, or production of bactericidal substances.

Schwartz et al. have demonstrated that nicotine, a major component of cigarette smoke, reduced the pinocytotic activity of peritoneal macrophages (50), and enhanced the release of pinolysosomal contents (51). Although the mechanisms for these effects are not known these data suggest that smoke, its chemical components, or other air contaminants severely damage two mechanisms by which alveolar macrophages ingest a variety of substances.

C. Synthetic Activities

Yeager has demonstrated that acute in vitro exposure of M. bovis induced rabbit alveolar macrophages to aqueous extracts of cigarette smoke, or its gas phase, reduced the rate and amount of ^{14}C -leucine incorporated into acid insoluble protein (52). Similar observations have been made by Holt and Keast with short term exposure of cultured alveolar and peritoneal macrophages to whole smoke or its vapor phase (37). However, when cells were maintained in culture for 24 hours, after exposure to smoke, there was no significant difference between the incorporation of ^3H -leucine by smoke exposed or control cells. These data, which were interpreted as a "recovery" phenomenon, must be interpreted with caution because the ability of control macrophages, after 24 hours in culture, to incorporate ^3H -leucine was decreased by about 70%.

Chronic exposure of macrophages in culture to whole smoke ($\frac{1}{2}$, 1, or 2 puffs each day for 4 consecutive days) resulted in a marked dose-dependent response in RNA and protein synthesis (37). At low doses ($\frac{1}{2}$, or 1 puff) there was a significant increase in both RNA and protein synthesis; the highest exposure always resulted in a depression of incorporation and viability. The authors claimed that chronic exposure to low doses of smoke lead to the production of metabolically activated cells, and suggested that an increased incorporation signified an adaptative response of cells to a toxic environment. Earlier studies of Holt and Keast demonstrated a marked increase in the incorporation of ^3H -uridine into peritoneal and alveolar macrophage RNA thirty minutes after exposure to whole smoke or its gas phase (35). Two patterns of nucleic acid synthesis were observed in macrophages 24 hours after exposure: 1) when more than 20% of the cells were dead, there was a marked increase in incorporation of uridine; and 2) when fewer than 20% of the cells were dead, tritiated RNA synthesis was significantly decreased. These authors suggested that the susceptibility of macrophages (measured by viability and changes in RNA synthesis to cigarette smoke) may be determined, in part, by their basal rate of RNA synthesis.

There are several alternative interpretations to the data of Yeager and Holt and Keast: 1) the decrease in incorporation of labeled amino acids or nucleic acid precursors may reflect a decreased permeability of the macrophage membrane or a decreased activity of membrane transport systems for these substrates; 2) the highly significant increases in protein and nucleic acid synthesis observed after short or long term exposures may result from a transient increase in permeability, carrier activity or cellular metabolism; 3) the depression in incorporation of the labeled

precursors may also reflect alterations in RNA or DNA polymerase activity, stability of messenger RNA for the protein being synthesized, ribosomal stability, or pool sizes of the substrates; 4) uniformly labeled uridine is not an accurate index of RNA synthesis because uridine can be metabolized to cytidine, and incorporated into DNA; 5) "rate" of incorporation implies that the time course of the incorporation was studied when, in fact, Holt and Keast used only a single time of incubation; 6) dead cells, estimated by trypan blue uptake, were not removed from the incubation medium before measurement of incorporation, and one would expect the reported data represented an "average" incorporation by damaged and undamaged cells.

Neither Yeager or Holt and Keast considered these alternative interpretations and until such parameters of substrate incorporation are evaluated the data presented can not be precisely interpreted.

D. Enzyme Activities

The plasma membrane of macrophages plays an important role in the specialized activities of phagocytosis, pinocytosis, chemotaxis and active transport. Migration of macrophages, engulfment of particles, or transport, requires expenditure of energy, and a mechanism for converting the energy of ATP into the mechanical events of translocation. In many cellular systems, an ATP-ase mechanism has been implicated as the membrane bound enzyme system responsible for transduction of chemical energy into work (53). Cross et al. have demonstrated Na^+ , K^+ , Mg^{++} adenosine triphosphatase activity in plasma membranes of sheep alveolar macrophages (54). Since this enzyme system is membrane bound and an integral part of the ion "pump mechanism," it would appear to be useful as a 'marker' for quantitative studies on the interactions of air contaminants with membranes of cells in the lung. Cigarette smoke is known to contain a number of substances which could inhibit the activity of the membrane ATP-ase system (27). Nicotine (54) and cadmium (56), two components of cigarette smoke, have been shown to inhibit the activity of this enzyme system in alveolar macrophages. Although these studies did not establish a relationship between inhibition of this enzyme system and other functions of membranes, they certainly suggest an attractive hypothesis for evaluating the toxicities of air contaminants.

Recent experiments of York et al. demonstrated an inhibition of cellular respiration by extracts of smoke (59). Since the alveolar macrophage is an obligatory aerobe (58), these studies point out another potential mechanism for the toxic effects of cigarette smoke; that is, inhibition of aerobic metabolism would lead to a deficiency of ATP which, in turn, could produce a depression in phagocytosis, substrate transport, or synthetic activity.

E. Amino Acid Transport in Alveolar Macrophages Exposed to Air Contaminants

Previous studies in this laboratory have demonstrated that aqueous extracts of cigarette smoke (AECS) or nicotine, at concentrations which did not significantly reduce cellular viability, as measured by dye exclusion, markedly depressed the transport of

alpha aminoisobutyric acid (AIB) by rabbit alveolar macrophages (59). Since this material has been submitted for publication, a summary of the significant observations is included here.

Incubation of macrophages with 0.01, 0.05 or 0.1 ml of AECS for 60 minutes resulted in a small but not significant increase in AIB accumulation as measured by intracellular to extracellular ratio greater than controls (Table I). However, higher concentrations of AECS always resulted in a highly significant decrease in accumulation of AIB. The stimulation of membrane transport probably resulted from an increased metabolism; the depression in transport at the higher concentrations is probably related to an alteration in carrier activity of generation of ATP by the cells because there was no significant loss of cells or a marked reduction in cellular viability (Figure 1).

AECS (0.5 ml) depressed the rate of accumulation and steady state value of AIB in alveolar macrophages. Since maximal and initial rates of transport, as a function of substrate concentration, were not measured, I could not determine if the capacity of the system, or the nature of the carrier systems were altered by AECS (Figure 2).

Incubation of alveolar macrophages with nicotine, a major component of cigarette smoke, and known to be present in the extract, produced a biphasic response in the accumulation of AIB (Figure 3). Interestingly, the concentrations of nicotine, which produced a significant depression in AIB accumulation, have been previously shown to markedly inhibit cellular respiration and the activity of sodium, potassium, magnesium, ATP-ase in alveolar macrophages prepared from sheep (55, 56). Preliminary studies with cadmium also reveal a similar biphasic effect on the active accumulation of AIB by rabbit alveolar macrophages.

My studies demonstrated that AECS, nicotine or cadmium can depress the accumulation of AIB by alveolar macrophages. The importance of these observations is not yet certain, but I believe these results clearly indicate that air contaminants, at remarkably low concentrations, can adversely affect specific and non-specific membrane functions of alveolar macrophages, and perhaps other cells in the lung. There are several possible mechanisms by which air contaminants induce alterations in plasma membranes. These include impairment of membrane synthesis, accelerated degradation of membranes, aberrant lipid and or protein synthesis, enzyme inhibition, or peroxidation of membrane lipids. With the exception of lipid peroxidation, there is no information available on the mechanisms by which air contaminants induce membrane alterations. Recently, Delucia et al. (60) have shown that exposure of rats to ozone, an oxidizing air pollutant, decreased the -SH content in membrane, protein, and nonprotein fractions of rat lungs. Although these measurement were not made with macrophages or other cells in the lung, oxidation of cellular reducing substances, such as sulfhydryl compounds, clearly does occur in lungs of experimental animals exposed in vivo to realistic concentrations of air pollutants. Recent studies in this laboratory have demonstrated the formation of lipid peroxides in alveolar macrophages after 60 minutes of incubation with aqueous extracts of smoke; addition of cysteine to the incubation media reduced the formation

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AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

Table I. α -aminoisobutyric acid transport in alveolar macrophages incubated in vitro with aqueous extracts of cigarette smoke.

| AECS ml | N | IC/EC (mean \pm S.E.M.) | % Change |
|------------|----|------------------------------|----------|
| 0 | 12 | 6.10 \pm 0.54 | |
| 0.01 | 12 | 6.32 \pm 0.97 | +7 |
| 0.05 | 10 | 6.16 \pm 1.09 | +4 |
| 0.10 | 8 | 6.93 \pm 1.18 | +17 |
| 0.25 | 10 | 4.16 \pm 0.65* | -30 |
| 0.50 | 9 | 2.70 \pm 0.53* | -55 |
| 0.80 | 8 | 1.59 \pm 0.38* | -74 |

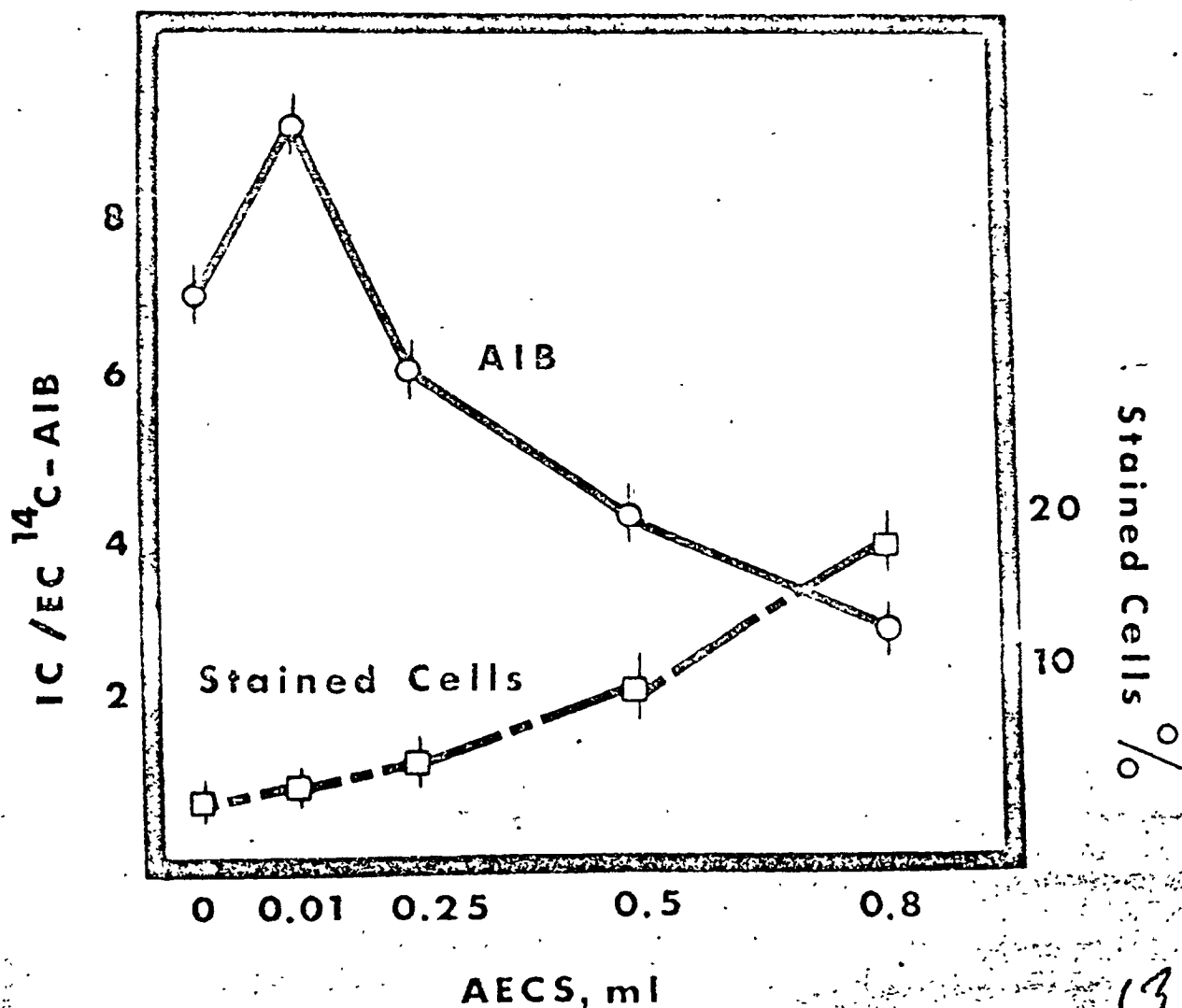
Rabbit alveolar macrophages were incubated with ^{14}C -AIB and various concentrations of aqueous extract of cigarette smoke for 1 hour as outlined in materials and methods. N is the number of experiments. * indicates significance of difference from controls calculated by students' "t" test $P \leq .001$.

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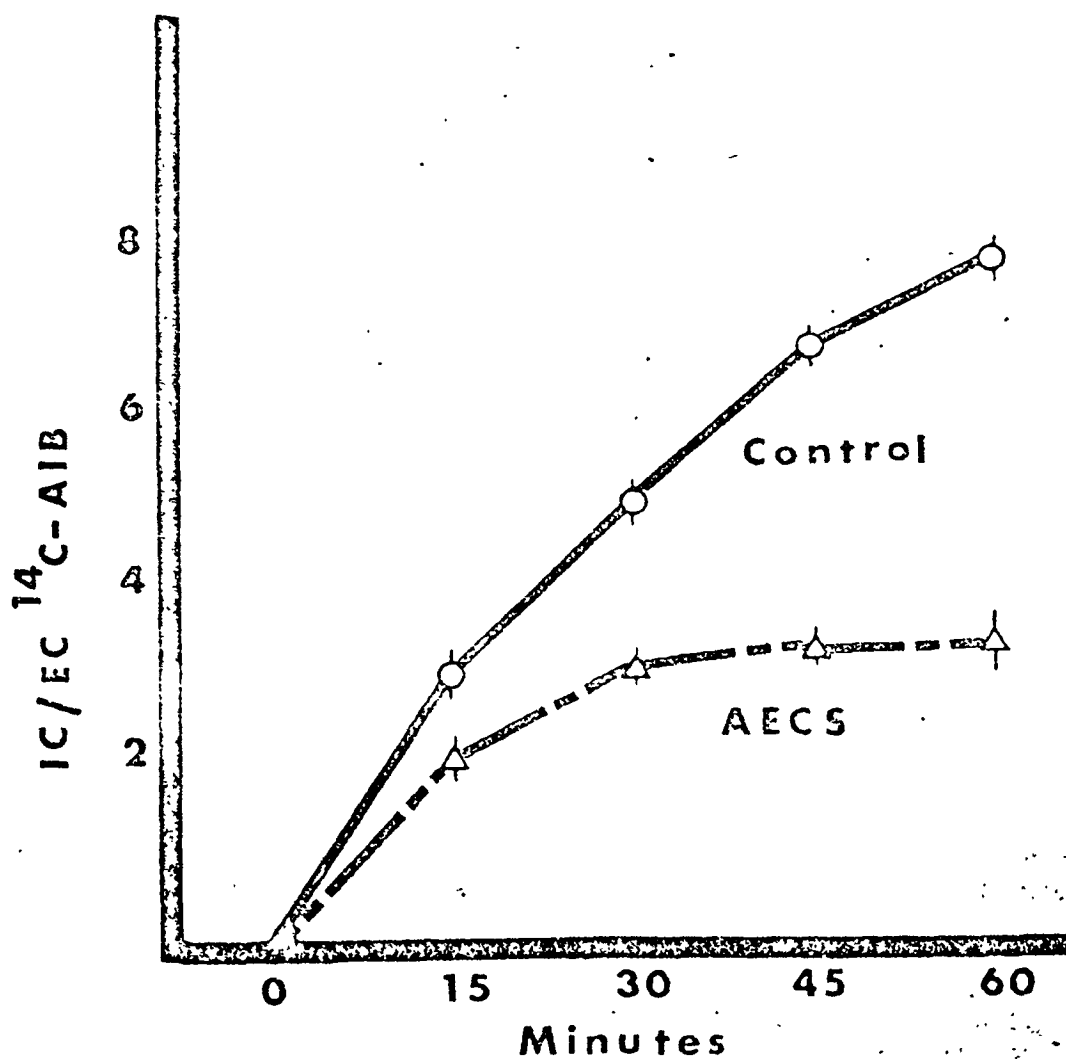
AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

Figure 1. Cells were incubated with ^{14}C -AIB as outlined in Materials and Methods. The percent of cells that stained with trypan blue was determined after one hour of incubation with the AECS (broken line). There was no significant change in the total number of cells in the incubation vessel after 60 minutes of contact with the extract. Each point is the mean and range of two experiments with duplicate samples at each concentration.



AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

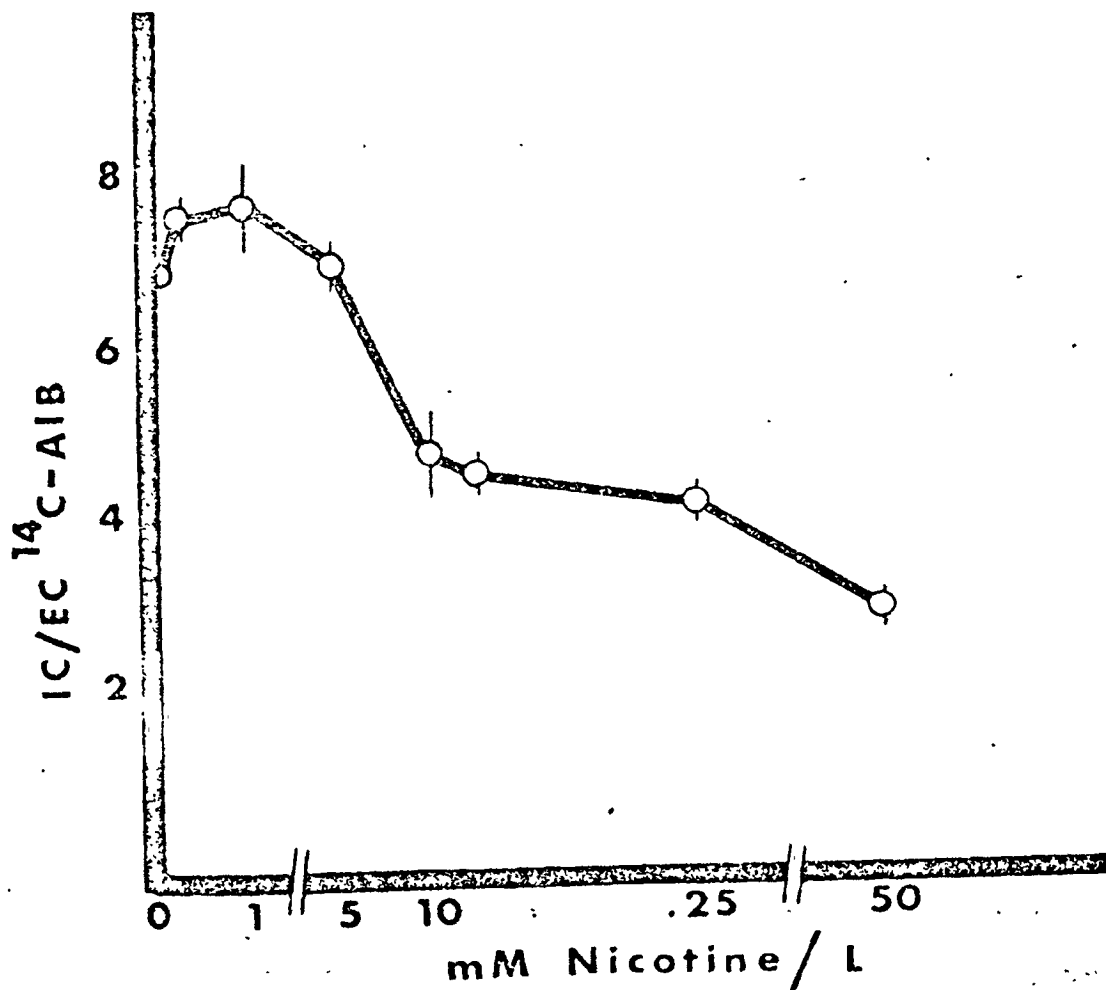
Figure 2. The effect of AECS on the time course of AIB uptake. Rabbit alveolar macrophages were incubated with ^{14}C -AIB for 15, 30, 45 and 60 minutes in the presence or absence of 0.5 ml of AECS. Less than 20% of the macrophages incubated with AECS took up trypan blue after 60 minutes of incubation. Each point is the mean and range of 2 experiments with duplicate samples



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AMINO ACID TRANSPORT BY ALVEOLAR MACROPHAGES

Figure 3. The effect of nicotine on the transport of AIB by rabbit alveolar macrophages. Alveolar macrophages were incubated with ^{14}C -AIB and various concentrations of nicotine for 1 hour. Each point is the mean and range of 2 experiments with duplicate samples.



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of peroxides (61). While these data are far from conclusive, they support the concept that oxidizing air contaminants can reduce the level of reducing agents in cells.

F. Rational For The Proposed Research

Numerous reports in the literature have described the effects of air contaminants on the phagocytic and metabolic activities of macrophages but there are no reports on the influence of cigarette smoke on membrane transport of specific substrates by alveolar macrophages. Preliminary studies in this laboratory have demonstrated that transport of α -amino isobutyric acid (^{14}C -AIB) by alveolar macrophages was adversely affected by concentrations of smoke extract, nicotine or cadmium which had little or no effect on cellular viability as measured by dye exclusion techniques. Since active transport of AIB appears to be a more sensitive and a less equivocal index of plasma membrane integrity than conventional dye exclusion techniques this research program shall investigate the mechanisms by which smoke inhibits active transport in alveolar macrophages.

I have chosen a non-metabolizable amino acid, α -amino isobutyric acid, as a substrate because it is known to be transported by alveolar macrophages (59), exhibits the transport characteristics of natural amino acids (62), and is not metabolized by the cell (63). Use of such a substrate enables membrane transport to be analyzed in absence of concomitant metabolic changes which are not directly involved in the transport process (67).

III. Methods of Procedure

A. Principle of the Assay System

The theoretical basis for employing active transport as an index of membrane integrity rests on the concept that normal cells will establish and maintain a concentration gradient of the substrate across the membrane (64). This concentration gradient is experimentally measured and expressed as an intracellular to extracellular distribution ratio. When the distribution ratio is greater than unity active transport has occurred; when the ratio is equal to unity no active transport has occurred and simple diffusion can account for the observed distribution ratio. In addition, kinetic analyses can identify the types of transport processes, and suggest whether an agent modifies the number or nature of the membrane carriers.

B. Type of Cells and Incubation System

Rabbit alveolar macrophages will be used because our previous studies (59) have used this macrophage and other investigators (24) have examined the types of transport systems existing in the membrane of the alveolar macrophage. In addition, the large number of cells which can be obtained from a rabbit are more than adequate for a single experiment. An in vitro system for exposing the cells to the test substances will be used because I wish to examine the

direct effects of cigarette smoke on the membranes of alveolar macrophages without the influence of other cell types or invasion of other cells into the lung in response to smoke. Additional advantages of in vitro exposures of homogenous cell suspensions over the intact tissue, such as lung slices, are: 1) faster and more complete contact of all cells with the test agents present in the incubation medium (this condition allows decreased reaction times and tends to enlarge the response); 2) a lower complexity of the biological model, represented by a two compartment system (cells, and incubation medium) which simplifies the analysis of the experimental observations, and the mathematical treatment involved.

It will be necessary to examine, in the future, the ability of cigarette smoke to alter amino acid transport after in vivo exposures. The primary purpose of these experiments is to examine the mechanism of action of AECS and specific components of AECS on amino acid transport systems in alveolar macrophages in a controlled manner.

C. Metabolic Substrate

^{14}C -AIB was selected as the substrate because it is known to be transported across the plasma membrane by an energy-dependent, concentrative carrier-mediated mechanism. Following uptake this analogue is neither incorporated into protein nor catabolized; hence its transport can be studied independently of complications introduced by such metabolism.

D. Selection of Test Agents

Three agents have been selected for evaluation on membrane transport: aqueous extracts of cigarette smoke (AECS), nicotine and cadmium. These agents will be used because my previous studies have shown that all three depress the accumulation of ^{14}C -AIB by rabbit alveolar macrophages. In addition, one might expect that water soluble compounds in smoke would dissolve in the aqueous phase covering the surface of macrophages as well as other cells in the lung. Nicotine and cadmium will also be used because both are found in smoke (27), are soluble in aqueous media, easily quantitated, and can depress the activities of sodium, magnesium, potassium activated adenosine triphosphatase localized in the membrane of alveolar macrophages (55, 56). However, nicotine and cadmium are only two of the many agents in smoke (27), and these choices should not be taken to represent a conclusion that they are the causal agents in the extract.

E. Technical Procedures

1. Preparation of Cells

Alveolar macrophages are obtained by the method of Myrvik et al. (65) as modified by Tsan and Berlin (24) to eliminate contamination with erythrocytes. Alveolar macrophages are sedimented from the lavage fluid by centrifugation at 2000 g-min at 4°. Cell pellets are resuspended in modified Hanks (MH) solution, pH 7.4, containing 5 mM glucose (24). Total number and viability of the alveolar macrophages are determined by incubation of cells ($1-2 \times 10^6$) in 2 ml of MH containing 0.1 ml of 0.5% trypan blue

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for 5 minutes at 22° and then counting the total number and the dye containing cells (30, 43). Approximately 1×10^8 alveolar macrophages with a viability greater than 95% are routinely obtained from a rabbit with this procedure. More than 90% of the cells so isolated are macrophages as identified by staining characteristics.

2. Preparation of Aqueous Extracts of Cigarette Smoke

Aqueous extracts of cigarette smoke (AECS) are prepared from 100 mm nonfilter, commercially available cigarettes with a Filtrona Smoking Machine (Cigarette Components, Ltd., Middlesex, England). I shall use an aqueous extract of cigarette smoke because previous studies (39, 49) have demonstrated that substances responsible for inhibition of phagocytosis, and killing of bacteria are located in the aqueous phase. Standard settings of a single 25 cc puff of two second duration once each minute are used in all experiments. Aqueous extracts of cigarette smoke are prepared by drawing the smoke from three cigarettes into 25 ml of ice chilled MH. All extracts are filtered through 0.45 μ millipore filter and adjusted to pH 7.4 with 0.1 N NaOH immediately before use.

3. Quantitation of Smoke Extracts

A common problem in examining the influence of gas phase smoke, whole smoke or aqueous extracts of smoke on cells is the absence of any quantitation of the smoke in the incubation media. In these studies the dry weight, and the concentrations of nicotine or cadmium will be determined in each extract. Dry weights of the extract will be made on samples of AECS evaporated to dryness at 45°. Alkaloids will be extracted from the extract with chloroform by the procedure of Welcher (66). This procedure, currently used in this laboratory, involves the extraction of alkaloids from pH 9 AECS and evaporation of the chloroform at 45°. Preliminary studies have revealed that 90% of the alkaloids are removed from the extract by two 10 minute extractions, with shaking, at room temperature.

The alkaloid residue is then dissolved in 0.5 N H_2SO_4 and the absorption at 259 m μ measured. The concentration of nicotine is then calculated from standard curves relating absorbance to concentration of nicotine.

Cadmium concentration in the AECS will be measured by atomic absorption spectrophotometry (67). Dr. Morris Spirtes, Adjunct Professor of Physiology, will perform these determinations.

These data on each extract will permit us to quantitate two of the components present in the extract, and allow us to determine if either agent is totally responsible for the observed depression in accumulation of ^{14}C -AIB by alveolar macrophages caused by AECS.

4. Amino Acid Transport

Alveolar macrophages, suspended in modified Hanks, are incubated at 37° in siliconized 15 x 150 mm glass culture tubes. Each tube contains 5×10^6 macrophages, 0.2 μ M ^{14}C -AIB, 0.2 μ Ci

^{14}C -AIB and modified Hanks in a final volume of 2 ml. All incubations are performed at 37° with gentle shaking (10-25 cycles/min) in a Dubnoff shaking incubator with air as gas phase. AECS, nicotine or cadmium in modified Hanks will be added at times and in amounts warranted by each experiment. All tubes are preincubated for 5 minutes before addition of the AIB to ensure temperature equilibration. At termination of the incubation, each cell suspension is transferred to a previously chilled tube and centrifuged at 2000 g- minutes. Each supernatant is transferred to a graduated tube, and the pellets washed twice with 2 ml of MH containing $0.1 \mu\text{M}$ ^{12}C -AIB/ml. The supernatants from each wash are combined with the first supernatant. Each cell pellet is resuspended in 0.5 ml distilled water (or 0.3 M KOH when protein is determined), transferred to a scintillation vial containing 10 ml Aquasol (New England Nuclear), and counted in a liquid scintillation counter. An aliquot of the thoroughly mixed supernatant is also counted to determine the amount of radioactivity remaining in the media after incubation. The efficiency of counting in each sample is determined by internal standardization using ^{14}C -toluene as standard, and the dpm calculated.

5. Estimation Of Intracellular and Extracellular Water Content

Replicate treated and control cell suspensions are incubated with ^{14}C -inulin in place of ^{14}C -AIB in exactly the same manner as that described for amino acid transport. The wet weight of the pellet is determined and the cell pellet dried at 80° for 18 hours. Total pellet water is calculated by subtracting the dry pellet weight from its wet weight. The dry pellet is then digested in 0.5 ml 0.3 M KOH and the radioactivity of the digested pellet determined. Extracellular water (ECW) is calculated by dividing the total pellet radioactivity by the radioactivity in the incubation medium. Intracellular water (ICW) is then calculated as the difference between total pellet water and extracellular trapped water. Our preliminary studies indicate that intracellular water represents about 70-80% of the total cellular weight. In these experiments we shall always determine if the test agents alter the ICW or ECW during the incubation period.

6. Calculation of Intracellular Amino Acid Content and Expression of Transport Data

The intracellular accumulation of ^{14}C -AIB per ml of intracellular water is calculated from a formula derived by Rosenberg et al. (68):

$$= \frac{R_t - A_o \cdot V_e}{V_t - V_e}$$

where R_t is the net dpm of cell pellet, A_o is the dpm/ml of incubation medium, V_e is volume of extracellular water in ml, V_t is total pellet water in ml.

Results are expressed as either the distribution ratio (A_i/A_o) where A_i is the dpm/ml cell water and A_o radioactivity/ml incubation medium or as umoles AIB per ml of cell water.

7. Measurement of Passive Diffusion, Kinetic Parameters of Transport and Efflux of AIB

The steady state distribution of AIB is determined by the rate of influx and efflux. As previously discussed net influx and net efflux are determined by two factors: passive diffusion and carrier mediated transport. The contribution of non-active transport (diffusion) to the accumulation of AIB is estimated by the method of Akedo and Christensen (62) which involves incubation of macrophages with high concentrations of substrate (40mM), at which the saturable component of AIB transport is presumably saturated. Correction for the diffusion component will be made in all situations in which the distribution ratio approaches 1.

The kinetic parameters of AIB transport are determined by measurement of initial velocity of uptake at different substrate concentrations. Suspensions of cells are incubated for 20 minutes with 0.1 μCi ^{14}C -AIB per ml and unlabelled AIB to give the following final concentrations: 0.1, 0.5, 1.0, 2.5, 5.0, 10, 25, 40 mM. Duplicate samples will be analyzed to obtain distributions ratios. The initial velocity (V_0) is given by the distribution ratio multiplied by the extracellular AIB concentration divided by the incubation time in minutes. The data are plotted as reciprocals of V_0 on the y axis, and reciprocals of substrate concentration on the x axis. In this presentation $\frac{1}{V_{\text{max}}}$ is given by the

intercept on the ordinate, and $-1/K_m$ by the intercept on the abscissa. Graphic presentation of initial velocity according to the method of Lineweaver and Burk, at AIB concentrations ranging from 0.1 to 40 mM will allow us to estimate the apparent affinity constant, K_m , and maximal velocity. Examination of these plots for linearity, will reveal if the transport of AIB is mediated by a single or multiple system. To confirm the kinetic constants obtained with the Lineweaver and Burk representation, these results will also be plotted as V_0 vs. V_0/S because this method has been found to give more reliable estimates of apparent K_m and V_{max} (69).

The rate of efflux of ^{14}C -AIB from alveolar macrophages which have been preloaded with ^{14}C -AIB is measured by determining the rate of loss of tracer from the cells into tracer-free external medium. The volume of the medium is relatively large so that the concentration of ^{14}C -AIB in the medium will be low throughout the experiment. Because of this recycling of the tracer into the cells should be negligible, and the rate of loss of tracer from the cells will be nearly equivalent to the rate of efflux.

Cell suspensions are preloaded with ^{14}C -AIB by incubation at 37° with 0.2 μCi ^{14}C -AIB and 0.2 μM ^{12}C -AIB. After 1 hour incubation the cell suspensions are washed three times with fresh medium and resuspended in 2 ml of AIB-free medium. After 5, 10, 15, 20 and 30 minutes of incubation at 37° duplicate samples are removed, centrifuged immediately to sediment the cells, and the supernatants assayed for radioactivity. The percent of radioactivity appearing in the medium is plotted against time of incubation to obtain an estimate of the amount of radioactivity which leaves the cell.

8. Estimation of ATP Levels in Alveolar Macrophages (19)

A 0.5-ml aliquot of each macrophage suspension is added to 4.5 ml of distilled water and placed in boiling water for 90 s, immediately chilled on melting ice and frozen in plastic tubes at -20°C for up to one week before assay. Macrophage ATP is measured by a modification of the firefly bioluminescence method. Disodium ATP (Sigma Chemical Co.) over a concentration range of 0.1 to 1 μM is used as standard. 0.1 ml of each thawed macrophage lysate, 0.85 ml of Tris-histidine buffer, pH 7.3, containing 80 mM NaCl, 15 mM KCl, 5 mM MgCl_2 , and 0.05 ml of luciferase previously prepared in distilled water are combined in scintillation counting vials and light emission measured exactly 30 s later in a Nuclear Chicago scintillation spectrometer. All measurements are made in triplicate or quadruplicate.

9. Determination of Sulfhydryl Levels (-SH)

SH levels in alveolar macrophages will be determined by the method developed by Sedlak and Lindsay (70), which determines the total -SH (TSH) in protein and non-protein compounds. The level of protein sulfhydryls is calculated as the difference between total -SH and non-protein sulfhydryls.

IV. Experimental Design

A. Time Course of Accumulation: Effects of AECS, Nicotine or Cadmium

These initial studies are designed to characterize the transport system by which alveolar macrophages accumulate AIB. In all experiments the viability of the macrophage suspensions (estimated by dye exclusion) and stability of cells (estimated by total cell number) will be measured to exclude an excessive number of dead cells or lysis as an explanation for the data.

The time course of AIB accumulation by alveolar macrophages in vitro will be determined by incubation of cell suspensions with 0.2 μM AIB including 0.2 μCi (^{14}C) AIB. Duplicate samples will be analyzed as described previously to obtain distribution ratios after 5, 10, 20, 30, 45, 60, 120, 180, and 240 minutes of incubation. Analysis of data, plotted as distribution ratio vs. time will identify the linear portion of uptake and the apparent steady state distribution ratio. Since alveolar macrophages also have systems which are neither concentrative nor energy requiring, I shall examine the non-active accumulation of ^{14}C -AIB. This passive diffusion component will be determined as described previously.

In order to determine if alveolar macrophages metabolize ^{14}C -AIB under the conditions of incubation used here, cell suspensions will be incubated with 0.2 μM AIB including 0.2 μCi ^{14}C -AIB for 60 minutes and the amount of $^{14}\text{CO}_2$ produced or the amount of ^{14}C amino acid recovered in acid precipitable protein determined. Additional studies will employ silica gel thin layer chromatography to determine if label is recoverable

in species other than ^{14}C -AIB, and to rule out the presense of ^{14}C -AIB in acid soluble proteins.

Since sodium ion is known to markedly influence AIB accumulation in a variety of cells, I shall determine the influence of extracellular sodium ion on the uptake of AIB. For these studies, cell suspensions will be incubated with ^{14}C -AIB for 1 hour in modified Hanks solution containing 36, 72, 144 mM NaCl. Choline chloride will replace NaCl in those solutions which contain less than normal Na^+ ions.

Next, the effect of extracellular pH on AIB accumulation will be measured. Cell suspensions will be incubated in modified Manks solution, pH 6, 6.5, 7.0, 7.4, 8, for 1 hour and the distribution ratios determined.

The energy dependency of AIB accumulation will be demonstrated by addition of sodium cyanide ($1 \times 10^{-4}\text{M}$), an inhibitor of oxidative phosphorylation, to the incubation media. This inhibitor should reduce but not completely abolish the accumulation of AIB by alveolar macrophages.

The kinetic constants of AIB transport by alveolar macrophages will be determined from measurements of initial velocity of uptake at different substrate concentrations as described in Section III E-7.

These control experiments will characterize the AIB transport system in alveolar macrophages and provide the baseline values for examining the effects of the test agents on membrane transport.

Our initial studies are designed to determine the dose response relationship of test agents on accumulation of ^{14}C -AIB by alveolar macrophages. The following parameters of transport: distribution ratio, apparent diffusion constant, K_D , and efflux of AIB, will be determined as a function of incubation time. The concentrations of test agents to be evaluated are:

AECS: 0, 0.01, 0.05, 0.10, 0.25, 0.5, and 0.8 ml/2ml

Nicotine: 0, 0.1, 1, 5, 10, 12.5, 25, 50 mM

CdCl_2 : 0.05, 0.1, 0.15, 0.2, 0.25, and 0.5 mM

Our preliminary studies have already demonstrated that .25 ml AECS, 25 mM nicotine and 0.15 mM CdCl_2 significantly interfere with AIB accumulation. However, these studies did not measure the influence of these agents on efflux of AIB or K_D and these alternate interpretations need to be examined.

From these data it will be possible to construct dose response curves, and these data will identify the concentrations of test agents which interfere with the accumulation of AIB or accelerate the exodus of AIB from the cells. It should also be possible to establish an effective dose (ED 50/60 concentration) for each agent. Dose response data will be analyzed (75) using a logarithmic

transformation for concentration of test agents and a probit transformation for distribution ratios obtained after 1 hour of incubation. A straight line fitted to the resultant points will permit estimation of the concentrations of test agents which reduce the accumulation of AIB by 50% in 60 minutes of incubation (ED 50/60). These data will facilitate comparison of the effective concentrations of each agent required for depression of AIB transport.

Next the influence of test agents on the time course of AIB accumulation will be determined. For these studies cell suspensions are incubated with the ED 50/60 concentrations of AECS, nicotine and cadmium for 5 to 240 minutes. Duplicate samples are removed after various periods of incubation and the distribution ratios determined. From the graphic representation of these data the apparent steady and the linear portion of AIB uptake can be estimated.

B. Effects of AECS, Nicotine, and Cadmium on Kinetic Parameters of AIB Transport

It is generally held that free amino acids are actively transported from the outer to the inner surface of the plasma membrane by a carrier-mediated process which derives energy from ATP. Our previous studies have shown that AECS, nicotine and cadmium impair active accumulation of AIB and these agents could, therefore, inhibit active AIB uptake by limiting the amount of ATP available to support transport or by modifying the nature of the carrier system. Accordingly, these agents might be expected to decrease the total capacity of the system, as estimated by maximal velocity measurements, or by decreasing the apparent affinity of the carrier for the substrate, as estimated by the calculation of K_m . Therefore, we shall examine the effects of the test agents on the following kinetic parameters of active transport: initial velocity, maximal velocity, and apparent affinity constant.

For these experiments cell suspensions are incubated with and without ED 50/60 concentrations of AECS, nicotine or cadmium for 15, 30, 60, or 120 minutes before measurement of initial velocities. After each interval of preincubation, cell suspensions are transferred to tubes containing 0.1 μCi ^{14}C -AIB/ml and unlabeled AIB to give a final concentrations of 0.1, 1.0, 5, and 10 mM. After 20 minutes of incubation, duplicate samples are analyzed, as described previously, to determine the distribution ratios. Graphic representation of initial velocity versus substrate concentration according to the method of Lineweaver and Burk will allow us to estimate the apparent affinity constant, K_m , and the maximal velocity, V_{max} . Examination of the plots for linearity will reveal if the transport of AIB is mediated by a single or multiple system and the type of inhibition observed. If treatment with the test agents depresses V_{max} , but fails to change the apparent affinity constant, K_m , these data will suggest a decrease in the total capacity of the system.

C. Relationship Between the Effect of AECS, Nicotine or Cadmium on AIB Transport and ATP Content of Alveolar Macrophages

If the experiments described in section 2 indicate that total capacity of the transport system is decreased by incubation of alveolar macrophages with ED 50/60 concentrations of test agents, I shall then examine the following hypotheses:

1. the test agents depress the active accumulation of AIB by reducing the supply of ATP for transport
2. a depression in ATP production will occur simultaneously with or before the depression in AIB accumulation

Cell suspensions are incubated with and without ED 50/60 concentrations of test agents for 5, 15, 30, 60, and 120 minutes before measurement of distribution ratios and cellular ATP levels. Duplicate control and exposed cell flasks are required at each time interval. These studies will indicate the temporal relationship between the level of cellular ATP and AIB accumulation; in addition, these studies will indicate if alterations in AIB transport precede or occur simultaneously with the reduction in ATP concentration.

ATP is required also for continual protein synthesis and these test agents may depress active amino acid accumulation indirectly by blocking synthesis of carrier proteins. Accordingly, we shall examine AIB transport and ATP levels in macrophage suspensions treated with test agents and cycloheximide alone and with both agents simultaneously. Previous studies of Baran (19) have shown that blockage of protein synthesis in thymic lymphocytes with cycloheximide inhibits amino acid transport, a finding which suggests that inhibitors of protein synthesis prevent replacement of labile proteins required for transport. If inhibitors of protein synthesis do not similarly decrease cellular ATP, these data will indicate that this protein is not required for ATP generation. In addition, this observation, which indicates that the effects of the test agents require continuing protein synthesis will be consistent with two possible modes of action: 1) the test agents selectively inhibit the formation of a labile protein involved in transport, or 2) the test agents promote de novo synthesis of inhibitors of transport or a fraudulent protein.

D. Relationship Between the Effect of AECS, Nicotine or Cadmium on AIB Transport and Modification of the Carrier

If the kinetic studies carried out in B suggest that the apparent affinity constant, K_m , of the transport system is altered by incubation of alveolar macrophages with the test agents, we shall examine the effects of these agents on the total sulfhydryl levels in alveolar macrophages and attempt to correlate this factor with alterations in AIB transport.

Since Tsan and Berlin (17) have demonstrated that the carrier system for lysine is especially sensitive to a sulfhydryl inhibitor which binds exclusively to membrane -SH groups, we shall attempt

to demonstrate inhibition of AIB transport by PCMBSA. Cell suspensions are incubated with various concentrations of inhibitor (0.001 to 1 mM) for 20 min, rinsed twice, and then the rate of AIB transport determined. If these experiments reveal PCMBSA inhibition of AIB transport, it is probable that SH are required in the active site of the AIB carrier.

Since -SH may be an integral part of the carrier system, we shall determine the levels of nonprotein and protein sulfhydryls in alveolar macrophages incubated with and without ED 50/60 concentrations of the test agents. Cell suspensions will be incubated with test agents for 15, 30, 60 or 120 minutes before determination of distribution ratios and SH contents.

These studies should indicate if there is a temporal relationship between levels of sulfhydryls and AIB transport. If such a relationship is suggested, an important mechanism of action of these three test agents will be apparent.

E. Reversibility of the Effect of AECS, Nicotine or Cadmium on Membrane Transport of AIB

These studies will examine the persistence of the depression in AIB transport induced by the test agents. Macrophages will be incubated with and without ED 50/60 concentrations of test agents for a period of time known to reduce AIB transport. Then the cells will be washed and re-incubated in the absence of the test agent for various intervals of time (15-240 minutes) before measurement of AIB transport, cellular levels of ATP and total sulfhydryl content. These studies will indicate whether the macrophages can recover their ability to transport AIB and the time required for repair.

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#61 - MICHAEL

1003540114

February 7, 1974

Grant application No. 961

CHRONIC PULMONARY DISEASE

To: The committee comprising Drs. Liebow, Meier and Wyatt

Subject: Dov Michaeli, Ph.D., Univ. of California School of Medicine, San Francisco
New application No. 961
"Effects of Cigarette Smoke on Pulmonary Fibroblasts and Collagen
and its Relation to Emphysema"

History

This proposal is a successor to CTR grant No. 786 "Collagen Antibodies in Relation to the Etiology of Emphysema", under the direction of Drs. Fudenberg and Michaeli, funded for three years ending March 31, 1974.

Request

Application No. 961 requests \$96,603 plus two additional years.

Document Submitted

Attached is application dated 1/10/74 (35 pages). The last two pages are a progress report on the predecessor grant.

Comment

Enclosed is a memo from Dr. Gardner on his visit to this laboratory, July 10, 1974.

FWN:gh

Encls.



F.W.N.

1003540115

July 24, 1973

MEMORANDUM FOR FILE

SUBJECT: Grant 786R2.

H. Hugh Fudenberg, M.D.

"Collagen Antibodies in Relation to the Etiology of Emphysema."

Site Visit July 10, 1973.

Dr. Fudenberg had a pile of manuscripts, reports and reprints assembled and we went too rapidly down the stack.

First, he made it clear that he wanted a smoke delivery machine. They could use it for in vitro and in vivo experiments. They had some in vivo studies completed. Smoke was bubbled through media and added to fibroblast cultures. It caused the cells to round up but not necessarily die. They, I believe, lost their collagen synthetic capacity. Collagen synthesis in vitro was followed by H³ or C¹⁴ proline incorporation. S³⁵ was used for hyaluronic acid. Acetaldehyde in amounts possibly comparable to smoke delivery amounts produced the same effects on fibroblasts. He was vague about quantitation or I was vague on uptake but 1/8 cig. per 1 ml media or equivalent to 1/8 was the figure I recall.

Chemically he has overcome the lack of solubility difficulty by different digests, pepsin digests, cyanogen bromide disassociation, SS fractures and obtained a¹, a² and b chains. Some amino acid sequencing has been done. He is comparing collagen from emphysematous lungs with that of normal lungs and fetal lungs and dermal collagen, fetal and adult. Collagen in different tissues differs, probably a greater organ specificity than species specificity. Seems to be different collagenesis in different tissues.

Fudenberg is keeping away from mice because of the histo-compatibility antigens which complicate the immunology probably more than HLA antigens.

Fine structure. He showed me many fine scan, freeze, et al., and freeze fracture ems. of the collagen of lung and tail. A manuscript has been submitted to J. Ultrastructure giving a somewhat new idea of collagen structure. Photographs of composite chains, fractures, cross-linkages (3 propyl nitrile) treated. Em. photo of emphysematous lungs seems to show consistent changes.

Phagocytic activity. Type 1 cells were shown in em. with large vacuoles containing one or more rbc, sometimes also in vacuoles and partially resolved. Mitochondria of these cells showed mitochondrial increase in number and size. Some studies were being done using cortisol and insulin.

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The studies on patients have now reached 150, about half of the proposed number. This will be completed during the year. Fudenberg aware of the limitations of genetic analyses by [IA] and developing a method of computer analyses of enzymatic inhibitions.

A Dr. Warner from Australia who has done much work on mouse antigens is going to be in the laboratory until January. Dr. Wells would like to stay one more year. Wells is working on the CEA specificity and has apparently found a specific antigen for lung cancer.

Fudenberg is planning to do more on cell immunity and is preparing fluorescence and rhubidium label materials - antigens - for such studies.

Fudenberg gave me two manuscripts and will shortly send more preprints. He may contact us about Wells. I'll be interested in seeing the work on tumor specific lung cancer antigens.

W.U.G.

WUG:ek

1003540117

#1961

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

JAN 30 1974

Application for Research Grant
(Use extra pages as needed)

Date: 1/10/74

1. Principal Investigator (give title and degrees):

Dov Michaeli, Ph.D. Assistant Professor of Biochemistry & Surgery
Co-investigator: H. Hugh Fudenberg, M.D., Professor of Medicine,
Professor of Bacteriology and Immunology

2. Institution & address:

University of California San Francisco
School of Medicine, Room 839 HSE
San Francisco, California 94143

3. Department(s) where research will be done or collaboration provided:

Departments of Biochemistry and Surgery and the Department of
Medicine

4. Short title of study:

Effects of Cigarette Smoke on Pulmonary Fibroblasts and Collagen
and its Relation to Emphysema

5. Proposed starting date: 3/1/74

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

1. Study the immunogenicity of cigarette smoke components complexed with lung collagen by the radioimmunoassay (RIA) technique.
2. To study the effects of components of the gaseous phase of cigarette smoke on the division rate of lung fibroblasts, and on the synthesis and secretion of collagen and mucopolysaccharides.
3. Identify the cellular site(s) of action of cigarette smoke components
4. Study the chemical interactions between cigarette smoke components and matrix macromolecules, and the possible effect of such interactions on the biosynthetic activity of lung fibroblasts.
5. Study the immunopathology of emphysematous lungs using immuno-electronmicroscopic techniques.

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8. Brief statement of working hypothesis:

2.

We have found (1) that approximately 70% of emphysema patients had antibody titers to denatured human collagen. In our search for a possible mechanism for the induction of such antibodies it became necessary to examine the effects of cigarette smoke under controlled conditions. We therefore developed a tissue culture system of human lung fibroblasts (WI-38) exposed to various concentrations of smoke components. We observed that at low concentrations (0.108 $\mu\text{g/ml}$) formaldehyde stimulated the synthesis and secretion of collagen and hyaluronic acid. At higher concentrations, formaldehyde caused a marked decrease in synthesis of connective tissue macromolecules. The mechanism for these effects may be due to a direct effect on the fibroblast and/or on the matrix macromolecules, which in turn causes an aberration of the signals received by the cells from the extracellular environment.

A major hurdle in studying the effects of cigarette smoke on fibroblast in culture under controlled environmental conditions was the inadequacy of currently available assays for measuring the low concentrations of newly synthesized collagen. We have recently solved this problem by developing an extremely sensitive radioimmunoassay assay technique (2). This makes the stated aims of this proposal extremely feasible and realistic.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Immunogenicity of collagen following its interaction with smoke components

The breakdown of tolerance to self-proteins through their chemical modification is a well-established phenomenon. Weigle (6,7) injected modified rabbit thyroglobulin into rabbits and created an autoimmune thyroiditis resembling Hashimoto's disease in humans. The possibility that interaction of cigarette smoke components with lung collagen may induce an immunologic reaction against this organ is very attractive. We have described the presence of antibodies to denatured collagen in 70% of emphysema patients, by the hemagglutination technique (1). However, these antibody titers may actually reflect a cross reaction of antibodies whose homologous antigen is lung collagen modified by smoke components. In order to evaluate this possibility, a much more sensitive and quantitative assay was required. Hence, a major research effort was invested in developing a RIA for collagen. We recently succeeded in perfecting such an assay and are capable of measuring 10^{-9}g (nanogram) quantities of collagen. This gives us the capability to measure:

1. the amount of circulating collagen in sera of emphysema patients, compared to normal sera and sera of other lung diseases, and
2. measure the amount of antibodies in sera of emphysema patients directed against lung collagen and against lung collagen modified by cigarette smoke components.

If indeed such a reaction can be demonstrated, isolation and identification of the newly created antigenic determinants will be of prime interest. The best approach would be to cleave the collagen with CNBr (8) and test the peptides for RIA. The significance of the immunological studies in general and the identification of the antigenic determinants in particular, will be discussed below.

2. Effects of cigarette smoke on lung fibroblasts

This study is already well underway. The following is a representative example of results obtained thus far.

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9. Experimental design and procedures (continued)

Cigarette smoke from one non-filter cigarette was passed through 50 ml. of Dulbecco media. The media was then diluted to dilutions ranging from 1:5 to 1:10,000. Assuming 15 puffs/cigarette, then 5 ml (the volume used in tissue culture dishes) of undiluted medium represents 1.5 puffs ($\frac{15 \times 5}{50}$), and diluted media represents a proportionate fraction thereof.

When lung fibroblasts (WI-38) were added to smoke-treated media many of the cells failed to attach to the dish. After a 2 day growth period the cells were pulse labeled with H^3 -thymidine and the amount of radioactivity uptake was determined. The results (Table I) indicate that extremely minute quantities of cigarette smoke can inhibit completely the mitotic activity of lung fibroblasts.

When treated media was added to established cultures, the cells were more resistant to cigarette smoke but were still substantially affected by the media.

Table I

Effect of media treated with cigarette smoke on division activity of human lung fibroblasts (WI-38)

| Treatment | Dilution of treated media | H^3 -thymidine uptake (cpm) | % inhibition |
|---|---------------------------|-------------------------------|--------------|
| I Control (untreated media) | - | 5801 | 0 |
| II Fibroblasts added to smoke treated media | 1 (undiluted) | 72 | 98.8 |
| | 1:5 | 331 | 94.3 |
| | 1:10 | 1666 | 71.3 |
| | 1:50 | 3335 | 42.6 |
| | 1:100 | 5738 | 1.0 |

Our next step was to incorporate components of the gaseous phase of cigarette smoke in tissue culture media and add it to established fibroblast culture. The parameters measured were multiplication rate (measured by incorporation of H^3 -thymidine), synthesis, and secretion of collagen (measured by RIA), and synthesis and secretion of mucopolysaccharides (measured by incorporation of $S^{35}O_4^{2-}$ or Cl^{14} sodium acetate). In general, formaldehyde exhibited the most profound effects with acetaldehyde next and propionaldehyde exhibiting only slight effects. No effect was exhibited by acetone, butanone and nicotine, at the concentrations tested. The concentrations added to the tissue culture medium were based on Kensler and Battista's analysis (2) of

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the composition of the gaseous phase of cigarette smoke. The results (Table 2) indicate several facts.

First, the injurious effects of aldehydes are in inverse relationship to the length of the alkyl group. Thus, although formaldehyde occurs in cigarette smoke in much lower concentrations than acetaldehyde, the former is by far the most injurious both relatively (per mole or μg) and absolutely (per cigarette). Secondly, close examination of the data reveals an interesting relationship between dose of aldehyde and its effect of fibroblasts. When fibroblasts were added to formaldehyde-or acetaldehyde-treated media, many cells either failed to attach or were more sensitive to the toxic effects of aldehydes after their attachment; this is reflected in a progressive decrease in the protein content/culture dish. The surviving cells in the formaldehyde-treated media also synthesized less collagen. A situation that much more closely resembles conditions prevailing *in vivo* occurs in treatment II, where aldehyde-treated media is added to established and confluent fibroblast cultures. At low levels of formaldehyde ($0.108 \mu\text{g/ml}$, equivalent to a concentration present in 0.03 puffs of cigarette smoke) there was an increase in total protein (122% of control) and a marked increase in collagen synthesis (255% of control), while no change occurred in the synthesis of mucopolysaccharides. At a higher concentration ($1.08 \mu\text{g/ml}$) the toxic effects of formaldehyde supervene and there is a general decrease not only in total protein (reflecting the number of surviving cells) but also in the synthetic rate of the surviving cells. These results provide only a glimpse of the potential that our tissue culture system provides for elucidation of biochemical mechanisms involved in the formaldehyde effects on cells, and for quantifying the dose-response relationship.

A third point of interest in our data is the effect of acetaldehyde. Although it is present at much higher concentrations in cigarette smoke, its effects on lung fibroblasts are much milder. Because of that, higher concentrations of acetaldehyde ($43 \mu\text{g/ml}$), added to confluent cultures, activate whatever mechanisms are responsible for the shift toward increased synthesis of collagen ($> 1000 \text{ ng}$) to an even higher degree than formaldehyde did. The significance of this observation in understanding the pathophysiology of certain fibrotic reactions in the lung are discussed below.

An interesting observation which is outside the realm of this application, is the progressive increase in thymidine uptake (i.e. proliferation rate) of fibroblasts when added to media containing acetaldehyde (Table I, treatment I). Is there a possibility that continuous maintenance of cells in contact with acetaldehyde would result in the eventual "escape" of some cells from the internal controls on proliferation rate, leading to an uncontrolled, neoplastic growth? Such a study would require long term experiments which are not contemplated in this proposal.

These results are probably merely two of the multitude of parameters that distinguish between the effects of moderate and high concentrations of formaldehyde and other aldehydes in cigarette smoke. Two of the most important parameters that we plan to measure are: 1) Effect of aldehydes on cell

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TABLE 2

| Treatment | Agent | Conc. µg/ml | Puffs equiv. | Protein | | Thymidine Uptake (cpm) | | | Collagen Synthesis (ng) | | | MPS Synthesis (cpm) | | |
|--|--------------|----------------|-----------------|---------|--------------|------------------------|------------|--------------|-------------------------|------------|--------------|---------------------|------------|--------------|
| | | | | Total | % of Control | Total | µg Protein | % of Control | Total | µg Protein | % of Control | Total | µg Protein | % of Control |
| I. Control (fibroblasts added to normal media) | - | | | 612 | | 3250 | 5310 | | 258 | 421 | | | | |
| Fibroblasts added to treated media | Formaldehyde | 0.011 | 0.003 | 504 | 82.3 | 3149 | 6248 | 117.6 | 197 | 390 | 92.6 | | | |
| | | 0.108 | 0.03 | 568 | 92.8 | 3212 | 5654 | 106.4 | 110 | 193 | 45.8 | | | |
| | | 1.08 | 0.3 | 60 | 9.8 | 181 | 3023 | 56.9 | N.D.* | | | | | |
| | Acetaldehyde | 0.432 | 0.003 | 588 | 96.0 | 3565 | 6062 | 114.1 | 147 | 250 | 59.3 | | | |
| | | 4.32 | 0.03 | 584 | 95.4 | 4012 | 6069 | 129.3 | 148 | 253 | 60.0 | | | |
| | | 43.2 | 0.3 | 340 | 55.5 | 3017 | 8873 | 167.0 | 127 | 373 | 88.5 | | | |
| II. Control (normal media added to confluent fibroblast culture) | - | | | 752 | | 990 | 1316 | | 320 | 425 | | 8177 | 10,873 | |
| Treated media added to confluent fibroblast culture) | Formaldehyde | 0.108 | 0.03 | 920 | 1223 | 1120 | 1217 | 92.4 | 1000 | 1087 | 255 | 10,062 | 10,966 | 101 |
| | | 1.08 | 0.3 | 444 | 59.0 | 628 | 1414 | 107.4 | 138 | 310 | 73 | 3,773 | 8,497 | 78 |
| | Acetaldehyde | 4.32 | 0.03 | 944 | 125.5 | 952 | 1008 | 76.5 | 250 | 264 | | 9,993 | 10,565 | 197 |
| | | 43.2 | 0.3 | N.D.* | | 735 | | | > 1000 | | | 7,624 | | |

* Not Done

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survival; (we already have qualitative observations that increasing concentrations of aldehydes cause increased cell death). 2) Effect of aldehydes on the length of fibroblast cell cycle. For this purpose it is imperative to work with synchronized cultures, and recently we have started such a culture using methods we used previously for synchronizing lymphocytes in in vitro culture (21).

3. Cellular sites of action of smoke components

These studies are designed to identify the mode(s) of action of aldehydes and other smoke components on the cellular level.

Labelled aldehydes will be added to the culture media and incubated with the cells for various time intervals. The labelled media will then be removed and the cells incubated with the corresponding unlabelled aldehyde to chase the unbound labelled agent. Cells will then be fractionated (22) and the various fractions counted.

We have already begun these experiments and preliminary results indicate that the cell membrane is the primary site of accumulation of formaldehyde; after 30 minutes of incubation 14% of the incorporated radioactive material was found in the membrane fraction, and after 24 hours incubation 40% was found in that fraction. These results are preliminary and a thorough study of all cell organelles and fractions, obtained by differential centrifugation in a sucrose gradient, is planned.

4. Interaction between cigarette smoke components and extracellular matrix

It is well known that the biosynthetic activity of mesenchymal cells is controlled to a large extent by signals from the extracellular environment (for review see 3). One of us (Z.N.) has demonstrated (4) that the concentration of chondromucoprotein in the matrix controls the rate of further synthesis by chondrocytes. We have recently been engaged in investigating the extracellular signals controlling the rate of synthesis of collagen and hyaluronic acid. It is quite likely the chemical interaction between the various aldehydes in cigarette smoke and collagen might cause a distortion in the signals received by the cells. For instance, we have found that the rate of collagen synthesis is inversely related to the extent of collagen cross-linking in the immediate environment of the fibroblast. Since formaldehyde and acetaldehyde are known cross-linking agents, it is conceivable that their effect would be to reduce collagen synthesis by lung fibroblasts.

We are proposing to react collagen with labelled formaldehyde, so that the extent of interaction could be quantitated. Also, the degree of cross-linking will be measured directly. This will be done by denaturation of the treated collagen at 50°C for 10 minutes and chromatography on a CM-cellulose column, according to the method of Piez et al (5). The ratio of β components to α monomer chains will serve as an accurate index for the degree of cross-linking.

Lung fibroblasts will be incubated in media containing either H^3 -proline,

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C^{14} glucosamine, or $S^{35}O_4^{=}$, and in the presence of collagen cross-linked to various degrees. The rate of collagen synthesis will then be measured by the rate of H^3 HyPro secretion into the media and/or by a RIA of collagen developed in our laboratory; the rate of sulfated mucopolysaccharide synthesis will be measured by the rate of C^{14} or S^{35} Hyaluronic acid secretion. Thus, this experiment, for the first time, will enable us to correlate the extent of collagen cross-linking with the rate of fibroblast synthetic activity, and more important for this proposal--will provide a basis for quantitation of the exposure to cigarette smoke and the effects of such exposures on the synthesis of new lung tissue.

5. Immunoelectronmicroscopy

Electron microscopic studies during the past three years (summarized in the Progress Report) indicate that profound ultrastructural changes occur in lungs of patients with emphysema (12) and in rodents with experimental emphysema (13) induced by antigens which inhibit collagen cross-linking. Common to and prominent among these changes in both man and rodent is loss of organization of collagen fibrils as shown by freeze-etch and freeze fracture electron microscopy.

To ascertain whether these changes are accompanied by and presumably due in part to immunologic events, antibody localization studies at the ultrastructural level are necessary to show that antibody to antigen are present at the site of the lesion. Such studies will also prove helpful in confirming any differences in antigen density found by the immunofluorescent technique (vide infra).

Lung tissues from smokers and non-smokers, emphysematous and "control" subjects will be obtained from pulmonary resection for other causes, and from autopsy. Tissue from animals with emphysema induced by β aminopropionitrile (13) will be obtained. With anti-collagen-anti-ferritin hybrid antibodies the relative numbers of native collagen/altered collagen sites per cell can be obtained, and correlation attempted with severity of disease (as measured by various pulmonary function tests), age, smoking history, and other parameters.

Comparable studies in β aminopropionitrile-induced emphysema in rodents, if producing identical results, will almost unequivocally demonstrate this to be a valid animal model for pulmonary emphysema.

Antibodies to human IgG, to collagen and to ferritin have already been prepared. Antibody to aldehyde-collagen complex is in the process of being prepared.

Hybrid antibody (one end directed to ferritin, the other end to IgG or to collagen) will be prepared by the method of Fudenberg, Drews, and Nisonoff (14).

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Addition of ferritin-collagen hybrid antibody to cells will result in the anticollagen site of the divalent antibody binding to the tissue, and the other site remaining exposed at 180 angstrom from the tissue antigen site; this is the best antibody method available for localization and enumeration of antigen sites per cell and has been used, for example, by Hammerling, et al (J. Exp. Med., 128, 1461, 1968), to enumerate the number of and to localize the sites of, the antigens in mouse thymocytes. It is vastly superior to use of native antibody labelled with ferritin or horse-radish peroxidase. Use of hybrid antibody to ferritin and to IgG will permit enumeration of the number of IgG molecules bound/cell. If these are all antibodies to native or all to aldehyde-collagen complexes, the number of molecules detected with the anti-IgG--anti-ferritin hybrid should be identical. If a portion of the emphysematous lung sites react with the anti-native collagen, and another portion with anti-aldehyde-collagen complex, data on relative ratios in various patients will be correlated with severity of disease (assessed by pulmonary function tests), duration of disease, smoking history, etc.

Significance

Achievement of the goals that we have set to ourselves will result in a better understanding of the relationship between cigarette smoke and emphysema. It may also offer a new approach to a sorely lacking means of early detection of the disease. Specifically, the four broad lines of investigation proposed herein have the following goals:

1. The study on the effects of various concentrations of aldehydes on cell division, life span, and synthetic activity may provide insights into the etiology and pathophysiology of emphysema. The slow disappearance of connective tissue components from the affected lung may be the result of normal turnover without the proper replacement. Such a process would result from killing of fibroblast and, at higher concentrations of formaldehyde, reduction of the synthetic activity of the surviving cells. Moreover, the observation that moderate levels of formaldehyde can cause an increase in the synthesis of collagen may explain the epidemiologic observation that among workers exposed to cotton dust (9) or to asbestos fibers (10), pulmonary fibrosis is more frequent and more extensive when cigarette smoking is added to the dust exposure.
2. Identification of the cell fraction(s) to which aldehydes bind will lay the groundwork for understanding the mode of action by which they exert their injurious effects.
3. The study of the interaction of smoke components with lung matrix molecules and study of the immunogenicity of these complexes may provide a basis for understanding the immunogenicity of lung collagen in emphysema patients, as illustrated by the fact that 70% of these patients have antibodies against collagen. This may also serve as a new approach to development of a test for early detection of emphysema. A key requirement for the latter two goals

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is the availability of a sensitive and quantitative assay for collagen. This we have already accomplished by the development of radioimmunoassay of collagen.

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Fluorescent Antibody Studies

(In contrast to the main body of the application, part of which has already been carried out, this part is more speculative and we are unsure about its outcome. We therefore propose it as a separate part, and are asking for only minimal funding to try it. If the idea proves successful, an expanded proposal along this line of investigation will be submitted.

Background

As stated previously in this application, we have found antibodies to collagen in 70% of patients with emphysema. If 1-5% of patients with emphysema develop the disease as a result of homozygosity for a gene producing protein inhibitor (alpha-antitrypsin, anti-collagen, and anti-elastin) deficiency, this leaves another 25-29% unaccounted for, suggesting that in the subject with normal Pi levels at least two separate etiologic mechanisms exist.

Objective

We propose to ascertain whether the two types are due to (a) differences in immunologic host defense, and/or (b) to differences in antigen content per cell in the pulmonary parenchymal cells in these two types.

(We also propose to investigate lung tissues of smokers and non-smokers (obtained during pulmonary resection for other causes, with autopsy) to discern whether antigen content/cell in smokers without emphysema differs from the normal non-smokers and from smokers with emphysema.

Further, although anti-collagen antibodies are presumably the result rather than the cause of the emphysema, it seems possible that once present, they may contribute to further lung damage in a genetically predisposed host, thus creating a vicious cycle. This also will be investigated.

Materials and Methods

The major difficulties with fluorescent microscopy (as ordinarily used) as applied to studies of the lung are:

- 1) Fixation of lung tissue for adequate spatial localization.
- 2) Inability to quantitate the intensity of fluorescence.

During the past two years, we have:

- (
- 1) Developed techniques for fixation of lung tissue for immunofluorescence and immunoelectronmicroscopy without loss of spatial relationship.
 - 2) Developed a technique, utilizing a Leitz Orthoplan fluorescent microscope with incident lighting and an MDVI Spectrophotometer

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with automatic read-out. This technique allows us to localize and quantitate (0-100% rather than 1+ to 4+) antibodies directed against single cells in tissues, (by automatic subtraction of background fluorescence) and to estimate differences in antigen density between cells from homologous tissues (16).

Antibodies to native collagen and denatured collagen have already been proposed, and those to collagen altered by various components of cigarette smoke are being proposed. These have been and will be labelled with fluorescent isothiocyanate and rhodamine by the methods used previously (17). All antisera will be characterized as to the peptide in collagen which bear the corresponding homologous antigenic determinant.

Pulmonary cells from non-smokers and smokers "normal", and from smokers and non-smokers emphysematous lung, will be compared with various antibodies by indirect immunofluorescence. Direct fluorescence will also be done to ascertain anti-collagen antibody bound in vivo.

Significance

1) Increased antigen density as revealed by the same antisera presumably renders a cell more liable to destruction by antibody than one with decreased numbers of antigen sites. Cigarette smoke aldehydes may pathologically act not only to cause destruction of pulmonary cells, but perhaps also to revive "buried" determinants by disabling a portion of the cell membrane (similar to the action of various proteolytic enzymes in exposing additional antigens on the red cell membrane (e.g. 18, 19). Similar studies will also be performed on cell lines of normal lungs in an in vitro culture before and after exposure to various cigarette aldehydes.

If such is indeed the case, antigenic density/cell in emphysematous lung as compared to that of non-emphysematous lung should be greater. Such comparison will be especially useful in lung tissue obtained from emphysematous and non-emphysematous subjects with comparable smoking histories matched for age, sex and ethnic origin.

If "normal" smokers, with few exceptions, show no differences as compared to normal non-smokers, perhaps a prognostic tool can be devised to predict those liable to develop emphysema (on the basis of similar studies on cells obtained from bronchial aspirators for cytologic studies), and thus council only those liable, presumably for genetic reasons, to develop the disease to avoid cigarette and other possibly deleterious environmental factors.

2) Alternatively, various aldehydes found in tobacco smoke, may alter collagen to produce neoantigens not present normally. Emphysematous lung may have a higher altered antigen density, and it might be possible to detect antigenic differences by immunofluorescent techniques between the normal and altered collagens and antisera from emphysematous animals or patients. The addition of antisera to cell lines, cultured with aldehydes

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in order to produce altered collagen, may prove of great value in establishing which immunologic factors contribute to destruction of such cells, and if so, which aldehydes are intimately involved.

In vivo investigation of antibody will also be studied; such localization would provide important evidence that the antibodies to native or to altered collagen, though previously a result of the underlying process, bind to pulmonary cells in vivo, thus producing a vicious cycle by causing further damage. Antisera to complement components C_4 , C_3 , and C_2 (20) will also be used in these studies, since simultaneous binding of anti-collagen immunoglobulins (Ig) and of complement would strongly suggest a cytolytic action in vivo.

1003540129

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10. Space and facilities available (when elsewhere than item 2 indicates, state location): Our biochemical laboratory has a total area of 1000 sq. ft. which includes 60 sq. ft. of office space. In addition, we have a tissue culture unit on an area of 150 sq. ft. Animals are maintained in central quarters, serving the whole institution. Major items of equipment include: 1) Centrifuges; low speed (International), medium speed (Servall) and an ultracentrifuge (Beckman, Model L). 2) Fraction collectors (Buchler refrigerated, LKB), columns, recorders (Leeds & Northrup). 3) Spectrophotometer, Beckman Model DB, used for continuous flow monitoring of column effluents. 4) LKB column effluent monitor, used for proteins other than collagen. 5) Liquid scintillation counter, Packard. 6) Disc gel electrophoresis apparatus, analytical (Canalco). 7) Auto-analyzer (Technicon), used for automatic analysis of hydroxyproline. 8) Other standard equipment of a biochemical laboratory (balances, desk-top centrifuges, autoclave, etc.). 9) Fully equipped tissue culture unit (incubator, UV hoods, microscope, microphotography equipment).

11. Additional facilities required:

NONE

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached Pages 20 and 21

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

| | | |
|--|------|-----------|
| Michaeli, Ph.D., Assist. Prof. Res. | 5% | ** |
| Nevo, Ph.D. Assist. Res. Biochemist III | 100% | 18,515.00 |
| J. Belton, Ph.D., Assoc. Res. Immunol. I | 35% | 7,537.00 |

Technical

| | | |
|---|------|-----------|
| D. Aiken, Staff Res. Assoc. III Step 5 | 100% | 16,712.00 |
| H. Scheuenstuhl, Staff Res. Assoc. III Step 5 | 100% | 16,712.00 |
| S. Inguito, Laboratory Assist. I | 50% | 4,858.00 |
| N. Parkin, Secretary II Step 5 | 25% | 2,677.00 |

**Salaries include 15% fringe benefits.

Sub-Total for A 67,011.00

B. Consumable supplies (by major categories)

| | |
|---|----------|
| Radioactive materials, solvents for assays etc. | 2,000.00 |
| Tissue culture media, dishes, gas mixture | 4,800.00 |
| Glassware and chemicals | 2,000.00 |
| Animals, purchase and maintenance | 3,000.00 |

Sub-Total for B 11,800.00

C. Other expenses (itemize)

| | |
|--------------------------------|----------|
| Travel (Aspen Emphysema Conf.) | 800.00 |
| Publication costs | 1,000.00 |

Sub-Total for C 1,800.00Running Total of A + B + C 80,611.00

D. Permanent equipment (itemize)

| | |
|--|----------|
| Spectrofluorimeter (for DNA Determinations) Turner Model 430 | 3,900.00 |
|--|----------|

Sub-Total for D 3,900.00

E. Indirect costs (15% of A+B+C)

E 12,092.00Total request 96,603.00

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|------------|
| Year 2 | 70,362 | 11,800 | 1,800 | -0- | 12,594 | 96,556.00 |
| Year 3 | 73,881 | 11,800 | 1,800 | -0- | 13,122 | 100,603.00 |

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Justification for Budget Items

Personnel

- Z. Nevo, Ph.D.--He is a highly trained biochemist and is well versed with tissue culture techniques which he learned in Dr. Dorfman's laboratory at the University of Chicago. He will conduct the biochemical assays on the cell cultures as detailed in this proposal.
- J. Belton, Ph.D.--Dr. Belton is an electron microscopist who, in addition to his skill with transmission electron microscopy, is familiar with the powerful techniques of freeze-fracture and freeze-etching electron microscopy.
- Technical:
- D. Aiken--Has participated in the development of a radioimmunoassay technique for determination of collagen and antibodies to collagen. She will be involved in application of this method as described in the proposal.
- H. Scheuenstuhl--Has been maintaining an excellent facility for tissue culture and will be indispensable for the continuation of the tissue culture studies proposed herein.
- S. Inquito--Will be involved primarily with dish washing and general maintenance activities. Tissue culture operations generate a large amount of glassware that has to be cleaned and sterilized, and her function in the smooth functioning of our studies is extremely important.
- N. Parkin--Will perform secretarial duties that have increased since we initiated the studies on effects of tobacco smoke on lung tissue and we anticipate a further increase in the load once the proposed projects proceed at full pace.

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Technician for Immunofluorescence

50%

5,226

As stated on page 8 of this application, the immunofluorescent studies are proposed on a trial basis. To carry them out, a technician for immunofluorescence will be needed at a 50% effort.

1003540135

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

| CURRENTLY ACTIVE | | | |
|--------------------------|--------------------------------|---------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| Career Development Award | 5 K04 AM50205 | 21,735 | 1/1/71-12/31/75 |
| Immunochemistry of Lung | HL-14759 | 250,000 | 3/1/72-2/28/76 |
| Collagen | GM18470 | 40,000 | 8/1/71-7/31/74 |
| Trauma Center Project | | | |

| PENDING OR PLANNED | | | |
|--------------------|--------------------------------|--------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| | | | |
| | | | |
| | | | |
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
The Regents of the University of California

Mailing address for checks

Gifts & Endowments
1487 4th Avenue
San Francisco, California 94122

Principal investigator

Typed Name Dov Michaeli, Ph.D.Signature Dov Michaeli Date 1/15/74

Telephone 415 666-1865
Area Code Number Extension

Responsible officer of institution

Typed Name Sue ClarkTitle Program Coordinator, Gifts & Endow-Signature Sue Clark Date 1/28/74 ments

Telephone (415) 666-2047
Area Code Number Extension

1003540136

Progress Report for CTR Grant #786

1. An important goal of this grant was to establish the role of the immune response against lung components in the etiology and pathophysiology of emphysema. We have found that 70% of patients with emphysema that we have tested had antibody titers against denatured collagen. This high incidence has great significance not only in understanding the pathophysiology of the disease but a possible means for early detection of the disease before respiratory symptoms become evident. Copies of the manuscript describing these results are attached.
2. Another important goal of this grant was to examine the hypothesis that aldehydes from cigarette smoke form a complex with lung connective tissue, specifically with collagen. Very early in the course of the investigation, we encountered difficulties in quantitating the titers to collagen-aldehyde complexes. This is because of the low degree of quantitation possible by the hemagglutination technique. We embarked on a program to develop a quantitative and sensitive technique to determine these antibodies. As a result of this investigation, we have developed a radioimmunoassay of collagen which enables us to detect nanogram quantities in the blood. This we consider a significant breakthrough in studies on collagen since collagen was impossible to assay in serum due to interfering substances. We have already started experiments to standardize the reaction between collagen reacted with radioactive formaldehyde and antibodies to collagen and to formaldehyde-collagen complex. This study is covered in the new grant proposal.
3. Experiments on the effect of aldehydes found in the gaseous phase of cigarette smoke on the proliferation rate and biosynthetic activity of lung fibroblasts have been undertaken recently and are described in detail in the proposal (pages 2-4). In short, we have found that minute concentrations of formaldehyde would cause an increase in collagen synthesis which in clinical situations may present as fibrosis. That this indeed is the case was determined by exposure of rats to low levels of formaldehyde. At higher concentrations aldehydes caused a marked decrease in both proliferative activity and biosynthesis of connective tissue. This observation may offer a rational explanation to the pathophysiology of emphysema. If indeed biosynthetic activity of lung fibroblasts is arrested, one would expect to see a slow disappearance of alveolar septal tissue. These experiments, although very exciting, are still in their initial stage and much more work in defining the molecular lesion is required.
4. Using freeze-etching and freeze-fracture techniques, we have characterized the ultrastructure of lung collagen (manuscript attached). We have now moved to the subject of the ultrastructure of lung collagen exposed to cigarette smoke. A smoking machine promised to us by Dr. John Kreisher of the CTR will be of tremendous help in this investigation.

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(A more complete report on the current status of our projects related to cigarette smoke is incorporated in the new grant proposal.

Supplemental

"A progress report on work that has little bearing on the present application will follow shortly".

1003540138

#938 - SACKNER

1003540139

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

October 19, 1973

Grant application No. 938

PULMONARY

To: The committee comprising Drs. Liebow, Sommers, Wyatt

Subject: Marvin A. Sackner, M.D., Mount Sinai Medical Center,
Miami Beach
New application No. 938
"Effects of Smoking on Mucociliary Clearance in Man"

History

This proposal was Case No. 215 and formal application was encouraged.

Request

Application No. 938 requests \$91,465 plus two additional years.

Documents Submitted (attached)

1. Application dated 8/15/73.
2. Consent form.
3. C.V.'s of Drs. Sackner, Landa, Wanner, and Michaelson.

FWN
F.W.N.

FWN:wg
Encls.

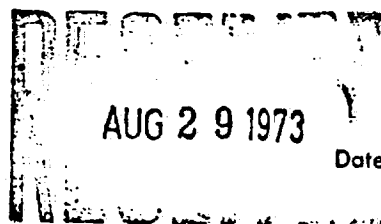
1003540140

#938

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)



Date: 8/15/73

1. Principal Investigator (give title and degrees):

Marvin A. Sackner, M.D.

2. Institution & address:

Mount Sinai Medical Center
4300 Alton Road
Miami Beach, Florida 33140

3. Department(s) where research will be done or collaboration provided:

Division of Pulmonary Diseases
Department of Internal Medicine

4. Short title of study:

Effects Of Smoking On Mucociliary Clearance In Man

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

1. To determine the acute effects of the smoke of tobacco products on tracheal mucous velocity in non-smokers and smokers.
2. To establish whether or not filters or additives to cigarettes such as menthol have a quantitative difference on tracheal mucous velocity during both short and long term exposure.
3. To perform tests of small airway obstruction in young smokers and to correlate the results of these tests with measurements of tracheal mucous velocity.
4. To test whether pharmacologic agents and environmental factors such as humidity and temperature modify the effects of smoking.
5. To collect and study the composition of mucus in anesthetized dogs by the methods of Proctor and his associates and to study the effects of smoking on changes in composition.
6. To develop lavage methods using the bronchofiberscope for collection of mucus and alveolar macrophages first in animals and later in man, after these methods are standardized, to study the effects of smoking on the various parameters.
7. To test the acute effects of smoking on viscosity of mucus and to measure the ciliary activity of ciliated epithelium by an in-vitro technique.

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2.

8. Brief statement of working hypothesis:

Although there is a great deal of evidence from the Surgeon General's Office that the smoking of tobacco products is associated with an increased incidence of pulmonary diseases, and that this information has been conveyed to the public, it has not cut down on the consumption of tobacco products. Many experienced physicians in the field of Pulmonary Disease recognize that it is impossible to prohibit or legislate bans on the smoking of cigarettes, owing to the fact that people simply enjoy this habit. However, it is important to distinguish characteristics among the various tobacco products that make them safer to the lungs. The mucociliary system constitutes an effective protective mechanism against inhaled particulate matter and its failure is often implicated in bronchopulmonary disease. Indeed, throughout the NHLI Task Force Report on Research in Pulmonary Diseases, it was emphasized that the study of pulmonary defense mechanisms, specifically, mucociliary clearance, was of prime importance if diminution in mortality and morbidity in lung diseases were to be achieved. Published reports on the effects of acute and chronic cigarette smoking on mucociliary clearance have been conflicting. Both increased and decreased clearance have been observed and undoubtedly some of the controversy has been related to the previous qualitative methodology for estimation of mucous clearance. Also, standardization of smoking in animals has been difficult and this may also have contributed to the difficulty in arriving at definitive conclusions. Recently we developed a cine-

(Continued on page 2A)

9. Details of experimental design and procedures (append extra pages as necessary)

1. A cine bronchofiberscopic technique for estimation of tracheal mucous velocity will be employed in paid volunteers and patients. If particles are placed on the mucosal surface, ciliary activity will carry them upward with the mucous blanket toward the larynx. Their movement can be filmed through a bronchofiberscope whose tip is located within the lumen of the airway of interest. Since the distal lens of this is wide angle and has an extremely small aperture, the image on the fibers is in satisfactory focus for object distances from 5 to 50 mm. The particles appear larger as they approach the lens of the bronchofiberscope. By standardizing the particle size and the film projection factor and knowing the filming speed, it is possible to compute their velocities from their projected image size.

In the absence of reference distances in the trachea, the distance of a Teflon disc from the distal lens of the bronchofiberscope must be determined from its image size in the projected film. Therefore, we employed a model of the trachea in order to establish the relation between the projected image size of the disc and its distance from the lens of the bronchofiberscope. The model trachea was mounted on an optical bench and a millimeter rule fixed within it. Teflon discs were placed at various distances between 20 and 50 mm away from the distal lens of the bronchofiberscope. The bronchofiberscope was centered within the model trachea so that the distal lens lay 8 mm radially from the millimeter rule. A 16-mm Beaulieu "Endo" motion picture camera was coupled to the bronchofiberscope and the illumination by the xenon light source (Olympus model CLX) set to "maximal brightness." Kodak Ektachrome (Daylight ASA 160) was used for documentation. The processed films were reviewed on a film viewer-reader (V/R 100-C/M Traid Corp., Glendale, Calif.). This rear projection viewer with a fixed magnification permits measurements of positions on the screen from mechanical counters coupled to manually controlled x and y cross hairs. The film frame may be read from a mechanical counter.

The cinefilms are viewed on the same film viewer-reader used for analysis of the films taken in the model trachea. Each particle whose velocity is to be computed is viewed at a minimum of three positions (frames) in the course of its filmed path. At each of these positions, the major axis of the elliptical particle image is located and measured by moving the cross hair intersection to the boundaries of this image on the axis. The disc diameter which forms the major axis of the image is always perpendicular to the line from the tip of the bronchofiberscope to the particle. For this reason, the major axis of the image depends only on the distance of the disc, not on the angle from which it is viewed. This increases the insensitivity of our method to decentering of the bronchofiberscope. Choice of frames

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9. Brief statement of working hypothesis (Continuation)

bronchofiberscopic method that can be employed in humans and animals to obtain serial measurements of tracheal mucous velocity over prolonged time intervals. A logical extension of our research activities is to study the effects of smoking on tracheal mucous velocity, to develop methods of collecting and analyzing mucus, to integrate histologic examination of ciliated epithelium and the alveolar macrophage in order to achieve a better understanding of the effects of smoking on these host defense mechanisms.

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9. Details of experimental design and procedures (Continuation)

from which image size is to be recorded must also be made in such a way as to isolate mucous motion from motion of the tracheal wall due to respiration and heartbeat. This is accomplished by choosing a fixed point on the viewing screen and taking measurements only from frames in which some tracheal landmark coincides with this point. Dark fixed spots due to broken optical fibers are readily available screen points and tracheal rings are convenient tracheal landmarks. If no dark spot falls on a tracheal ring, a contour is drawn with a grease pencil on the viewing screen to coincide with the tracheal ring in frames from which data are taken. All measurements for velocity computation are made at resting lung volume (FRC) position.

To compute a mean tracheal mucous velocity, 10-12 particles are measured in each run. We trace lines on the viewing screen to divide the trachea into four quadrants and try to measure image size of three to four particles in each quadrant.

- a. Acute effects of cigarette smoking. Ten light or nonsmokers and ten heavy smokers will be given cigarettes standardized for tobacco and nicotine content and will be asked to smoke them in a standardized fashion. Comparisons will be made on the acute effects of smoking different cigarettes on tracheal mucous velocity on separate days. Observations will be carried out over a 30 minute period and should mucous velocity be depressed, administration of catecholamines will be given in an effort to speed it up. These subjects will also be asked to abstain from smoking one to two weeks and the experiments repeated.
- b. Subjects who are known to have small airway obstruction (see screening tests for estimation of small airway disease) versus cigarette smokers matched for pack years but who do not have evidence of small airway disease, will be selected. Tracheal mucous velocity will be measured in both groups to ascertain whether impairment of this parameter is an earlier sign of pulmonary dysfunction than smaller airway disease. Attempts to modify mucous clearance will be accomplished by administration of catecholemines and other pharmacologic agents.
- c. The effects of preconditioning with air of low humidity or high humidity and high or low temperature will be studied to obtain an understanding of the interrelationship of environmental factors in the response to smoking. The subjects will be placed within an environmental chamber presently in our laboratory prior to smoking a cigarette and then tracheal mucous velocity will be measured.
- d. The effects of standardized cigarettes supplied by the Tobacco Industry will be employed to derive quantitative differences on tracheal mucous velocity among various brands of tobacco products.

2. Tests of small airway obstruction to correlate with measurements of tracheal mucous velocity. There has been a great deal of emphasis recently on the detection of small airway disease by means of various proposed tests. These include measure of closing volume, analysis of the flow volume curve, analysis of single breath nitrogen test, measurement of the frequency dependence and measurement of frequency dependence of lung compliance. The latter is thought to be the most sensitive technique and also is the standard for discrimination among normal and diseased subjects in the presence of otherwise normal pulmonary function. However, it is an invasive test involving the swallowing of an esophageal balloon and therefore is unsatisfactory for widespread screening. We have recently confirmed a mathematical link between frequency dependence of lung compliance and distribution of ventilation, the latter determined by the nitrogen washout technique. Assuming a two compartment system with equal compliances in making corrections for Pendelluft and common dead space mixing effects,

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9. Details of experimental design and procedures (Continuation)

the ratio of dynamic to static lung compliance for any respiratory frequency can be calculated from the compartmental analysis of the nitrogen washout at a single respiratory frequency. Using these equations, a good correlation was found between calculated and measured C_{LD}/C_{LS} in a mechanical lung model, in dogs with artificially induced bronchial obstruction, and in young smokers or young non-smokers following carbachol inhalation. Finally, a two compartment nitrogen washout was demonstrated in 10 healthy smokers at one or two respiratory frequencies whereas all 10 normal controls showed a single exponential curve. These findings indicate that the non-invasive nitrogen washout test is capable of predicting C_{LD}/C_{LS} and at the same time gives a direct measure of gas distribution. Further, it appears to be a highly sensitive method for the detection of "small airway disease".

As mentioned above, we will attempt to screen smokers with similar smoking histories for evidence of small airway disease and those with no evidence for this and ascertain whether mucous clearance parallels these tests, whether the suppression of mucous clearance is found only in those subjects with the small airway disease or whether it is not found at all. These patients will then be challenged with cigarette smoke to ascertain what happens to tracheal mucous velocity.

3. Collection and Composition Of Mucus In Anesthetized Dogs or Unanesthetized Sheep.

We have previously demonstrated that tracheal mucous velocity can easily be measured in anesthetized dogs. However, it is also possible to study unanesthetized sheep. We do not have facilities for housing sheep within our Animal Laboratory and we first plan to make these measurements in anesthetized dogs and later should they prove of interest in conscious sheep in an animal facility that will have to be rented outside the hospital. We intend to make measurements of the respiratory mucus after the method described by Proctor, Aharonson, Reasor and Bucklan: (Bull. Physio-Path. Resp. 9: 351-357, 1973). These authors placed a soft, plastic coated, glass fiber mesh into the upper trachea to collect mucus and an absorbent cotton wad just beneath the vocal cords. They found that the total tracheobronchial mucous flow seemed to be about 1 to 3 grams an hour. They collected the mucus from the mesh and the cotton and then washed the material from these collecting devices to study the composition of mucus. We plan to estimate the total amount of mucus as did Proctor and associates and also to measure the viscosity, total solids, nitrogenous components, electrolytes, and protein. We plan to study the acute effects of smoking and correlate it with tracheal mucous flow. Once the acute studies are completed, we then plan, some time after the initial granting period to study the chronic effects of cigarette smoking on these variables as to whether any adverse effects can be protected by the addition of additives to the smoke. For example, it has been shown by Dalhamn and Rylander, (Am. Rev. of Resp. Dis. 103: 855-857, 1971) that oxolamine citrate and Phenyl vinyl oxadiazole and Phenyl methyl oxadiazole when added to cigarette smoke prevent a depression of ciliary activity.

4. Lavage technique for alveolar macrophage using the bronchofiberscope. We are presently developing a technique using a silastic rubber balloon at the end of the bronchofiberscope to obtain alveolar macrophages by subsegmental lavage from different portions of the lungs. We intend to first develop this technique in anesthetized animals and later to use it in patients and normal volunteers. We shall correlate tracheal mucous velocity on the one hand and alveolar macrophage recovery and activity in response to the smoke of various tobacco products. By employing subsegmental lavage, different portions of the lungs can be sampled during a given study as a function of time. Harris, Swenson and Johnson (J. Clin. Invest. 49: 2086, 1970) have previously measured phagocytic activity and glucose

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9. Details of experimental design and procedures (Continuation)

utilization in human alveolar macrophages in smokers and nonsmokers. The macrophages were obtained by bronchopulmonary lavage and the studies were carried out in vitro in the absence of smoke. We plan to extend these studies by obtaining repeated subsegmental lavages as a function of time.

5. Estimation of viscosity of bronchial secretions. Although viscosity of sputum has been measured by many investigators over the past several years, results have been disappointing. This is because expectorated sputum is a nonhomogeneous material and therefore different samplings give widely varying values. This may also be true of bronchial secretions but no good data are available on this fluid owing to the prior difficulty of measuring the viscosity of a small sample. Recently, a new clinical viscometer has been designed which appears to circumvent these difficulties (Bleeker and Hoeksema: Ann. Otol. 82: 248, 1973). This device requires only .015 ml of specimen. We hope to obtain this quantity of mucus first from anesthetized dogs by developing a capillary meshwork device for sampling within the tracheobronchial tree. Later on, the mucus will be sampled for change in viscosity after acute exposure to different tobacco products.
6. In vitro determination of ciliary activity. Measurements of mucociliary clearance, as in our cine-bronchofiberscopic technique, give an overall picture of transport of mucus which is dependent on its composition, viscosity and amount, as well as on the integrity of ciliary activity. It is not possible to monitor ciliary activity in vivo but with exfoliated bronchial epithelium mounted in a nutrient wet preparation, preliminary microscopic studies indicate that ciliary activity can be measured using a visual auditory feedback technique (Bleeker, personal communication). We believe that a stroboscopic technique may be an improvement of this method for measurement of ciliary activity. We plan to develop this technique in animals and then apply it in humans smoking various tobacco products.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

On or about October, 1973 the Division of Pulmonary Diseases will be moved into a new area in the Blum Research Building, consisting of 8,000 sq. ft. Of this space, approximately 4,500 sq. ft. are devoted to laboratories for both clinical and research work. The clinical pulmonary laboratory is equipped with 1) two automatic pulmonary function devices, 2) three slowly responding helium analyzers and one rapidly responding helium analyzer, 3) three nitrogen gas analyzers, 4) two carbon monoxide infrared analyzers, 5) Pulmonet with closed circuit helium setup, 6) Collins body plethysmograph, 7) Ergometer, 8) DR-8 Electronics for Medicine oscilloscope with recorder and PR-7 recorder, 9) setup for rebreathing diffusing capacity, 10) four desk calculators, 11) one rapidly responding and two slowly responding oxygen analyzers, 12) two blood gas electrode machines, 13) one Capnograph, 14) one simulated breathing device, 15) Wolff 4 channel tape recorder. The research laboratory is equipped with 1) DR-12 Electronics for Medicine oscilloscope recorder, 2) Grass 78 - 13 channel recorder, 3) Phillips 7 channel analog tape recorder, 4) 12 channel Electronics for Medicine oscilloscope recorder, 5) horizontal body plethysmograph for combined cardiac and pulmonary studies, 6) vertical environmental chamber-body plethysmograph, 7) nitrous oxide infrared gas analyzer, 8) bag and box spirometers, 9) apparatus with associated hybrid computer for pulmonary and chest wall mechanics and for estimation of closing volumes, 11) Perkin Elmer mass spectrometer, 12) five Olympus bronchofiberscopes, two light sources for taking of cine film, and two Bealeu motion picture cameras. A biochemistry support laboratory is housed in this new facility. It is equipped for radioactive isotope waste disposal.

Computer support is from two PDP-12 computers. One PDP-12 computer has 12 K core, 800,000 word disc, hard wired floating point package and a Versitek line printer. The other PDP-12 has 12 K core and a card reader. Several software packages have been developed for pulmonary function testing and for research data processing.

11. Additional facilities required:

(Continued 3A)

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

(See page 3B and 3C)

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10. Space and facilities available (Continuation)

On the hospital grounds is an animal research laboratory with approximately 500 sq. ft. of space. Equipment includes 1) DR-12 oscilloscope recorder, 2) horizontal body plethysmograph, 3) image intensifier and x-ray equipment with cine, 4) nitrous oxide analyzer, 5) CO₂ analyzer, 6) TMC 7 channel tape recorder, 7) Gas Chromatograph, 9) LINC 8 digital computer. Adjacent to the animal laboratory is a trailer containing offices and a rear view motion picture projector with paper tape output for preparation of data for computer processing of mucous velocity experiments.

1003540148

13. Five most recent and pertinent publications:

Marvin A. Sackner, M.D.

1. Avery, W.G. and Sackner, M.A.: A rapid measurement of functional residual capacity in the paralyzed dog. J. Appl. Physiol. 33: 515-518, 1972.
2. Sackner, M.A., Wanner, A. and Landa, J.: Applications of bronchofiberscopy Chest, 62: Suppl. 2, 70S - 78S, 1972.
3. Michaelson, E.D., Sackner, M.A. and Johnson, R.L., Jr.: Vertical distributions of pulmonary diffusing capacity and capillary blood flow in man. J. Clin. Invest. 52: 359-369, 1973.
4. Sackner, M.A., Rosen, J.J. and Wanner, A.: Estimation of tracheal mucous velocity by bronchofiberscopy. J. Appl. Physiol., 34: 495-499, 1973.
5. Sackner, M.A., Rosen, M.J. and Wanner, A.: Effects of oxygen breathing and endotracheal intubation on tracheal mucous velocity of anesthetized dogs. Bull Physiol-path. Resp. 9: 403-415, 1973.

Jose F. Landa, M.D.

1. Landa, J., Avery, W.G. and Sackner, M.A.: Some physiologic observations in smoke inhalation. Chest, 61: 62-64, 1972.
2. Amikam, B., Landa, J., West, J. and Sackner, M.A.: Bronchofiberscopic observations of the tracheobronchial tree during endotracheal intubation. Am. Rev. Resp. Dis., 105: 747-755, 1972.
3. Sackner, M.A., Wanner, A. and Landa, J.: Applications of bronchofiberscopy. Chest, 62: Suppl. 2, 70S - 78S, 1972.
4. Sackner, M.A. and Landa, J.: Bronchofiberscopy: To intubate or not to intubate! Chest, 63: 302, 1973.

Adam Wanner, M.D.

1. Wanner, A., Zigelboim, A. and Sackner, M.D.: Nasopharyngeal airway: a facilitated access to the trachea. Ann. Int. Med., 75: 593-595, 1971.
2. Wanner, A., Amikam, B. and Sackner, M.A.: A technic for bedside bronchofiberscopy. Chest, 61: 287-288, 1972.
3. Sackner, M.A., Wanner, A. and Landa, J.F.: Applications of bronchofiberscopy. Chest, 62: 705-785, 1972.
4. Sackner, M.A., Rosen, M.J. and Wanner, A.: Estimation of tracheal mucous velocity by bronchofiberscopy. J. Appl. Physiol. 34: 495-499, 1973.
5. Wanner, A. and Sackner, M.A.: Transvenous phrenic nerve stimulation in anesthetized dogs. J. Appl. Physiol., 34: 489-494, 1972.

1003540149

13. Five most recent and pertinent publications (Continuation)

Edward D. Michaelson, M.D.

1. Walsh, R.E., Michaelson, E.D., et al.: Upper airway obstruction in obese patients with sleep disturbance and somnolence. *Ann. Int. Medicine*, 76: 185-192, 1972.
2. Michaelson, E.D., Sackner, M.A., and Johnson, R.L., Jr.: Vertical distributions of pulmonary diffusing capacity and capillary blood flow in man. *J. Clin. Invest.* 52: 359-369, 1973.
3. Crossley, R.J., Leverett, S.D., Jr., Shubrooks, S.J., Jr., Michaelson, E.D., and Burton, R.R.: Human physiologic responses to high, sustained +G_z acceleration. Preprints of Scientific Program, Aerospace Medical Association, May 7-10, 1973.
4. Shubrooks, S.J., Jr., Leverett, S.D., Jr., Crossley, R.J., Michaelson, E.D., and Burton, R.R.: Human physiologic responses to high, sustained +G_z with positive pressure breathing. Preprints of Scientific Program, Aerospace Medical Association, May 7-10, 1973.
5. Michaelson, E.D., O'Byrne, B.: Effects of varying inspired oxygen concentrations (FIO₂) on pulmonary mechanics. Preprints of Scientific Program, Aerospace Medical Association, May 7-10, 1973.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

| | % time | Amount |
|--------------------------|--------|--------|
| Marvin A. Sackner, M.D. | 15 | 0 |
| Adam Wanner, M.D. | 10 | 3,600 |
| Jose Landa, M.D. | 33 | 12,000 |
| Edward Michaelson, M.D. | 17 | 6,000 |
| Biochemist (to be named) | 100 | 18,500 |

Technical

| | | |
|-----------------------------------|-----|-------|
| Research Technician (to be named) | 100 | 9,500 |
| Pulmonary Technician | 30 | 0 |

Sub-Total for A 49,600

B. Consumable supplies (by major categories)

| | |
|--------------------------|-------|
| Cine Film and Processing | 4,500 |
| Glassware, disposables | 3,000 |
| Animals | 4,000 |
| Chemicals, reagents | 2,500 |

Sub-Total for B 14,000

C. Other expenses (itemize)

| | |
|--|-------|
| Fees for Volunteers (for bronchofiberscopic studies) | 8,000 |
| Travel | 500 |
| Publication Costs | 1,000 |

Sub-Total for C 9,500Running Total of A + B + C 73,100

D. Permanent equipment (itemize)

| | |
|--|-------|
| Viscometer for microanalysis of mucus (for measuring mucous viscosity) | 3,000 |
| Series H20 Advanced Phase Microscope Model H20 TG-P-8 with photographic accessories (for measurement of ciliary beating of bronchial epithelial cells) | 4,400 |

Sub-Total for D 7,400E 10,965

E. Indirect costs (15% of A+B+C)

Total request 91,465

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 52,328 | 14,000 | 9,500 | 0 | 11,374 | 87,202 |
| Year 3 | 55,206 | 15,000 | 9,500 | 0 | 11,956 | 91,662 |

1003540151

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---------|--------------------|
| Volumes And Compliance Of Pulmonary Circulation | NIH-HL 10622 | 200,825 | 9/1/66-8/31/75 |
| Effects Of Oxygen On Mucous Clearance Rates | NIH-71-2205 | 101,715 | 7/1/71-7/1/74 |
| Detection And Prevention Of Regional Pulmonary Atelectasis, Edema, And Hypoperfusion During Acceleration | Aerospace Med. Div. F 41609-72-C-004 | 98,832 | 10/14/71-10/14/73 |
| Pulmonary Training Grant | 1 TO1 NL5980-01 | 200,000 | 7/1/73-7/1/78 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--------------------------------|---------------------------|--------------------|
| Host Defense Mechanisms Of The Lung | NHLI | Application Incomplete | 5/1/74-5/1/77 |
| Detection and Prevention Of Regional Pulmonary Atelectasis, Edema, And Hypoperfusion During Acceleration | Aerospace Med. Div. | 49,900 | 10/14/73-10/14/74 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Marvin A. Sackner, M.D.Signature Marvin A. Sackner Date 8/15/73Telephone 305 538-6030
Area Code Number Extension

Checks payable to

Mount Sinai Medical Center

Mailing address for checks

4300 Alton RoadMiami Beach, Florida 33140

Responsible officer of institution

Typed Name Samuel GertnerTitle Executive Vice PresidentSignature Samuel Gertner Date 8/15/73Telephone 305 532-3611 3311
Area Code Number Extension

1003540152

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4975

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1974

Grant application No. 975

CHRONIC PULMONARY DISEASE

To: The committee comprising Drs. Liebow, Sommers and Wyatt

Subject: Herbert W. Wallace, M.D., Graduate Hospital of the Univ. of Pennsylvania
New application No. 975
"Pulmonary Biochemistry"

History

The Executive Committee initially considered this proposal as Case No. 212, and voted to encourage formal application.

Request

Application No. 975 requests \$60,201 plus two additional years.

Documents Submitted

Attached is application dated January 24, 1974.

Reprints of the publications listed on page 3A are available to you on request.

FWN:gh

Enclosures

FWM
F.W.N.

1003540154

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 4 1974

Application for Research Grant
(Use extra pages as needed)

Date:
January 24, 1974

1. Principal Investigator (give title and degrees): Herbert W. Wallace, M.D.
Associate Professor of Surgery
2. Institution & address:
Graduate Hospital of the University of Pennsylvania
19th and Lombard Streets
Philadelphia, Pennsylvania 19146
3. Department(s) where research will be done or collaboration provided:
Department of Surgery, Harrison Department of Surgical Research,
Department of Physiology
4. Short title of study: PULMONARY BIOCHEMISTRY
5. Proposed starting date: July 1, 1974
6. Estimated time to complete: Five years
7. Brief description of specific research aims:
 1. To determine the normal metabolic pathways and the primary substrates utilized as energy sources by pulmonary tissue;
 2. To determine the regulatory mechanisms of these metabolic pathways;
 3. To study the interrelationships between the metabolism of substrate and the synthesis of byproducts such as phospholipids and possibly prostaglandins;
 4. To evaluate the effects of tobacco smoke upon the metabolism of the pulmonary parenchyma;
 5. To compare the metabolic processes of animal experiments with those of normal human pulmonary tissue.

1003540155

Smoking has been accused of being a major causative factor in pulmonary cancer as well as a serious aggravating factor in other pulmonary diseases. However, most of the evidence for and against these accusations has been inconclusive and in many instances without good scientific basis. The purpose of this investigation is to elucidate the normal metabolic processes of pulmonary tissue and their regulation. Without these data, it is impossible to evaluate the metabolism or transport of other agents in pulmonary tissue and their relationship to metabolic alterations and neoplastic pulmonary disease. After completing baseline studies of pulmonary metabolism, we propose to evaluate the effects of tobacco smoke and/or its various components and attempt to elucidate these effects at a cellular level.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Background.1. Work accomplished by other investigators.

At the 1970 meeting concerning the morphology of experimental respiratory carcinogenesis sponsored by the NCI and the AEC, it was pointed out that a thorough understanding of the structural and functional components of the respiratory tract is necessary before a meaningful exploration of the causes of disease in this complex organ system can be undertaken. Biochemical and histochemical studies indicate that all major routes of carbohydrate metabolism appear to operate in mammalian lungs. These include the presence of the Embden-Meyerhof glycolytic pathway, the citric acid cycle, and the hexose monophosphate shunt. Several investigators, including Felts (1,2) have studied the utilization of lipids and their derivatives for synthetic purposes, but there is little clearcut evidence and much controversy concerning the role of lipids as an energy source for the lung. Studies on rats have indicated that lung slices can oxidize fatty acids (2,3). However, mitochondrial preparations of rabbit lungs reportedly have limited ability to oxidize β -hydroxybutyrate or other fatty acids (4). Although it is controversial whether lipid substrates are an important energy source for the lung, pulmonary tissues of all animals have been found to incorporate fatty acids for lipid synthesis (5,6). Oxygen consumption in the lung appears to be principally by the cytochrome chain, but there is some evidence that a substantial amount is used by enzyme systems that are not inhibited in the presence of cyanide in the incubation medium (7). However, it must be pointed out that under most circumstances of tissue incubation obtaining a complete block of oxidative phosphorylation by any of the known inhibitors can be difficult. Sorokin (8) has pointed out that pulmonary development can occur in vitro despite the presence of a high cyanide concentration in the medium; thus a noncytochrome-mediated oxygen metabolism might be involved in biosynthetic reactions. Dr. J. R. Vane (personal communication) of the Royal College of Surgeons points out that pulmonary tissue may have several functions besides that of transporting gases. He has shown that during perfusion of the lung with acetylcholine, bradykinin, 5-hydroxytryptamine and prostaglandins E_1 , E_2 and $F_{2\alpha}$ were apparently inactivated by pulmonary tissue, whereas other

(CONTINUED ON PAGE 2a)

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vasoactive amines, such as angiotensin II, adrenalin, oxytocin, vasopressin and histamine were not affected by passage through the pulmonary circulation. In both in vivo and in vitro animal studies he has shown that anaphylaxis, shock, and even ventilation cause the release of prostaglandin from the lung, which strongly indicates a biosynthetic process. The relationship of these metabolic processes to blood gas transport and pH would be interesting and important to elucidate. Recently, Holub (9), utilizing alveolar cells obtained from washings of rabbit lungs, demonstrated the possibility that these cells are involved in antibody formation. Some of these cells have also been shown to have phagocytic properties, which seem to be related to catalase-dependent peroxidative metabolism (10).

Much of the recent investigation has been focused on cells derived from lavage of the lung, particularly the macrophage. It has been suggested that the macrophage has a controlling role in carcinogenesis (11). The exact origin of the alveolar macrophages remains controversial. Some investigators, including Sorokin (12), believe that they originate primarily from lung tissue, while others, such as Pinkett et al. (13), have presented evidence in support of a peripheral hemopoietic tissue origin. Studies done by Massaro et al. (14) indicate that the alveolar macrophage is capable of synthesizing phospholipids and that glucose seems to be a source of energy for this process.

We originally became interested in these problems after reading a paper by Weber and Visscher (15), whose experiments showed that isolated, perfused lung produced a significant amount of lactic acid, even when the perfusate had a concentration of lactic acid greater than 100 mg/100 ml. These studies were done aerobically with excellent control of the perfusate, etc., and it was difficult to understand why an organ with the greatest access to oxygen of any in the body would need to produce lactic acid under aerobic conditions. Glaviano et al. (16) reported a significant pulmonary A-V difference of lactate, which appeared to increase with sympathetic stimulation. In 1958 Bucherl et al. (17) studied the A-V difference of lactic acid in 27 patients during cardiac catheterization and found a significant increase in lactic acid in the left atrial blood as compared to the pulmonary artery blood. Lochner and Nasserl (18) in studies of perfused lung demonstrated a lactic acid production of 4.78 mg/min per gm wet weight of tissue. In 1966 Salisbury-Murphy (19) utilized ^{14}C -labeled glucose at both the C-1 and C-6 positions and noted a CO_2 ratio for ^{14}C -1/ ^{14}C -6 of 2.5 for lung slices. In 1967 Wolfe et al. (20), utilizing isolated pneumocytes, found a C-1/C-6 ratio of 7. Both of these studies indicate a large hexose monophosphate shunt. Histochemical studies by Tyler and Pearse (21) revealed an absence of succinic dehydrogenase in alveolar cells. However, in the more recent work of Wolfe et al. (20) this enzyme was thought to be present. Felts (2) used evenly labeled ^{14}C -glucose with lung slices and observed that little $^{14}\text{CO}_2$ was produced, nor was much radioactivity found in the total fatty acids. The radioactivity that could be detected was primarily in the phospholipid fraction. In recent unpublished experiments my colleague Dr. Aron Fisher ventilated dogs using a tracheal divider. One lung was respired with room air and the other with 100% nitrogen for a 3-4 hour period. Subsequent electron microscopic studies of the lungs showed no alterations or differences between normoxic and hypoxic pulmonary tissue.

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Recent studies of lung mitochondria by Fisher et al. (personal communication; 22) suggest that little oxidative phosphorylation occurs. They feel that the moderate amount of ATP produced is utilized during phospholipid synthesis under the control of calcium ion.

All of the above data support our contention that the regulation of metabolic processes of lung tissue is unusual. Of interest and probable importance is the question, why does the organ with the greatest access to oxygen have to depend primarily upon anaerobic mechanisms and produce lactate (which it apparently is incapable of metabolizing efficiently). There are conflicting experiments with perfused lung (23), which, in contradistinction, showed no change or utilization of lactate in blood during passes through the lungs. Thus it appears that factors other than oxygen or substrate availability might control the oxidative metabolism of the lung.

It is of extreme interest, and perhaps of some importance, that many malignant tumors also have a high glycolytic capacity, as first described by Warburg. It is evident that the glycolysis is an important source of energy in cancer tissue, sufficient even for prolonged survival in the absence of oxygen. Thus, transplantable tumor may grow again after having been kept for three days in the absence of oxygen (24,25). This means that neoplastic tissues share with other tissues the capacity to obtain energy from two sources. The proportion of energy derived from glycolysis is very high in neoplastic tissue. The reasons for the excessive lactate production of neoplastic cells have only recently been elucidated. It is clear that extramitochondrial reduced pyridine nucleotides are not directly oxidized by the mitochondrial respiratory chain of animal cells. Lehninger (26) first observed that intact mitochondria isolated from liver were incapable of oxidizing added DPNH. Similar observations have been made with mitochondria of ascites tumor cells (27,28). The slow rate of penetration of pyridine nucleotides into liver mitochondria in the intact animal (12,29) confirms the conclusion drawn from the in vitro studies. It is now clear that an indirect mechanism by a shuttle system is utilized for the introduction of extra mitochondrial reducing equivalents to the mitochondrial respiratory chain. The high aerobic glycolysis in tumor cells has been ascribed to the lack of such a shuttle system (28,30,31). Among these systems, the α -glycerophosphate shuttle has been extensively studied, and a low activity of the cytosolic enzyme involved in this shuttle, the α -glycerophosphate dehydrogenase, has been considered typical of tumors with high rates of aerobic glycolysis (32). Such a defect may also be present in normal lung tissue, and, if so, would make this tissue exciting to study and compare with biochemical mechanisms occurring with neoplastic alterations. There are, of course, many other biochemical characteristics which distinguish neoplastic tissue from other tissue (33). Together, they are responsible for uncontrolled growth, but from a quantitative point of view, no single characteristic is as striking as the aerobic glycolysis.

2. Work accomplished by the applicants.

a. Lactate production of lung.

In 1958 Bucherl et al. (17) studied the A-V difference of lactic acid in 27 patients during cardiac catheterization and found a significant increase in lactic acid in the left atrial blood compared with the pulmonary artery blood. We repeated this study and obtained similar

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results (Wallace and Stein, unpublished data).

Using standard Warburg techniques, we incubated rat lung slices in Krebs-Ringer's phosphate buffer at pH 7.4 with 0.1% glucose and observed lactic acid production under aerobic (room air) and anaerobic (100% nitrogen) conditions for 30, 60, 90, and 120 minutes. Oxygen uptake and lactic acid production were found to be linear. In a series of 18 experiments the average oxygen uptake under aerobic conditions was 19.2 $\mu\text{l}/\text{mg}$ of nitrogen per hour. The lactic acid production of the tissue incubated with air was 37.5 $\mu\text{g}/\text{mg}$ of nitrogen per hour, while that of the tissue incubated anaerobically was 41.3 $\mu\text{g}/\text{ml}$ of nitrogen per hour, an insignificant difference.

In a series of pilot studies we repeated these experiments using cyanide as an electron transport inhibitor and again found that lactic acid production appeared equal under aerobic and anaerobic conditions. Because of the possibility that cyanide might interfere with the enzymatic determination of lactic acid through its interaction with pyridine nucleotides, we had attempted to remove the cyanide before conducting the analysis. Nevertheless, we felt that we would be on more secure ground if we used carbon monoxide. Therefore, we repeated these experiments under two conditions, using 1) 3% CO , 20% O_2 and 77% N_2 ; or 2) 100% CO , which was subsequently flushed out with the mixture of 3% CO , 20% O_2 and 77% N_2 . The oxygen concentration was checked with a Beckman oxygen meter. Control experiments with zero time incubation, etc., were carried out. The average values for lactic acid production under aerobic and anaerobic conditions with nitrogen, air and carbon monoxide were similar. Glucose uptake in air averaged 144 $\mu\text{g}/\text{mg}$ N_2/hr (15 experiments) and was similar with 100% CO (19 experiments).

These findings leave little doubt in our minds that lactic acid production is unaffected by the amount of oxygen available to the lung tissue. These preliminary data suggest that the major glucose pathway is not through the mitochondrial system. Another possibility is that the pathways of lactic acid production differ under aerobic and anaerobic conditions. Subsequent experiments will help to elucidate these problems.

In another experiment we used 0.1% glucose and 0.1% sodium acetate in the incubation medium. The sodium acetate was uniformly labeled with ^{14}C . A slight increase in lactic acid production was observed under anaerobic conditions as compared to aerobic conditions. These reaction mixtures were fractionated by a method similar to that of Massaro et al. (14). It is interesting to note that under aerobic conditions the radioactivity from the acetate could be found in relatively large amounts in the protein fraction and to some extent in the lipid fraction, whereas anaerobically following an aerobic incubation, the initial radioactivity seen in the lipid fraction seemed to decrease, possibly due to utilization of lipids as an energy source. Some of the radioactive CO_2 noted under these anaerobic conditions may have been due to decarboxylation. In an additional series of experiments, ^{14}C -labeled lactate was added as a substrate in the presence and absence of glucose aerobically and anaerobically. Almost no radioactivity could be found in either the produced CO_2 or any

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of the tissue that was analyzed, nor was there any detectable loss of ^{14}C -lactate from the incubation solution. The total lactate of the incubation medium increased during these experiments.

These preliminary experiments clearly indicate that rat lung slices are incapable of utilizing lactate as a substrate. In fact, the amount of lactate produced by the lung in the presence of excess lactate was unchanged.

In another group of experiments, ^{14}C -labeled octanoic acid bound to albumin (by the method of Millstein and Driscoll; 29) was employed as a metabolic substrate in the presence and absence of glucose anaerobically. No significant uptake of the fatty acid was detected, nor was radioactivity found in the collected carbon dioxide, indicating that this fatty acid is not a major energy source under these experimental conditions. Further experiments with palmitate are contemplated.

These data seem to indicate that the metabolic processes of pulmonary tissue are primarily an anaerobic process. The use of lung slices may be criticized, and our data are preliminary and incomplete and will require additional experiments to develop significant statistical data. However, they are supported by the recent studies of lung mitochondria by Fisher et al. (personal communication; 22) which suggest that little oxidative phosphorylation occurs. They feel that the moderate amount of ATP produced is utilized during phospholipid synthesis under the control of calcium ion.

b. Biochemistry of the tracheobronchial mucosa.

Many of the investigations of the effects of smoking on the tracheobronchial tree have been studies of ciliary action. Although the tracheobronchial mucosa has been pointed out to be one of the prime targets of the effects of smoking, in a careful review of the literature we failed to uncover any definitive biochemical studies of the mucosa of the tracheobronchial tree.

In an effort to obtain baseline values, we have performed preliminary studies on bovine tracheal and bronchial mucosa using standard Warburg techniques. In 85 experiments the average rate of oxygen uptake of cow tracheal mucosa was $30 \pm \text{S.D. } 8 \mu\text{l O}_2$ per hour per 100 mg wet weight, and the bronchi showed a similar uptake. A piece of human trachea obtained during a medically-indicated tracheostomy under anesthesia revealed an oxygen uptake of $16 \mu\text{l O}_2$ per hour per 100 mg wet weight. Two samples of human main bronchial mucosa obtained from fresh surgical specimens gave results of $19 \mu\text{l O}_2$ per hour per 100 mg wet weight. A piece of human lung was $14 \mu\text{l O}_2$ per hour per 100 mg wet weight. Utilizing ^{14}C -l-glucose, we also measured glucose consumption, CO_2 production and incorporation of radioactivity into lipids, protein and RNA. In six experiments glucose uptake averaged $4.9 \mu\text{g}$ per 100 mg wet weight per hour, with 41.5% of the metabolized glucose accounted for by CO_2 , 29.6% in inorganic lipids, 7.6% in RNA, and 21.3% in protein. Two experiments with human bronchial mucosa revealed a glucose uptake of $3 \mu\text{g}$ per 100 mg of wet tissue per hour, with 47% accounted for by CO_2 , 32% in protein, 18% in RNA, and 3% in inorganic lipids. Two pieces of normal human lung had an average glucose uptake of $40.5 \mu\text{g}$ per 100 mg wet weight per hour, with 67.8%

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TABLE 1. SUBSTRATE UTILIZATION

| ¹⁴ C label | Other substrate (1 mg/ml) | Oxygen utilization (μl/100 mg wet wt/hr) | Labeled substrate as precursor | | | | | | | |
|-----------------------|---------------------------|--|--|------------|--|-----------|--|--------------|--|-----------|
| | | | Total lactate production (μl/100 mg wet wt/hr) | | CO ₂ production (μmol x 10 ⁻³ /100 mg wet wt/hr) | | Lipid production (μmol x 10 ⁻³ /100 mg wet wt/hr) | | Protein production (μmol x 10 ⁻³ /100 mg wet wt/hr) | |
| | | | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| Glucose | None | 26±0.7 (107) | 82±6 (12) | 62±4 (12) | 28±1 (12) | 0 (12) | 2±0.2 (12) | 0.6±0.1 (12) | 4±0.2 (12) | 0 (12) |
| | Lactate | 22±2 (6) | 80±11 (24) | 80±12 (20) | 27±0.7 (5) | 6±2 (5) | 3±0.4 (6) | 0 (6) | 4±0.6 (6) | 0 (6) |
| | Acetate | 35±1 (12) | 70±0.3 (6) | 76±7 (5) | 32±4 (5) | 4±1 (5) | 4±0.3 (6) | 1±0.2 (5) | 2±0.3 (6) | 0 (6) |
| Lactate | None | 35±3 (6) | 6±17 (6) | | 201±14 (6) | 0 | 41±6 (6) | 0 (6) | 19±2 (6) | 0 (6) |
| | Glucose | 29±3 (6) | 94±21 (6) | 94±12 (6) | 124±26 (4) | 28±4 (6) | 5±1 (5) | 0 (6) | 18±2 (5) | 0 (6) |
| Acetate | None | 22±2 (6) | 3±0.8 (6) | 6±3 (5) | 73±14 (5) | 8±6 (5) | 2±0.5 (6) | 0 (6) | 9±3 (6) | 0 (6) |
| | Glucose | 36±3 (5) | 76±4 (5) | 85±7 (6) | 61±8 (5) | 6±1 (3) | 3±0.3 (6) | 0 (6) | 6±2 (6) | 0 (6) |

Values are mean ± standard error of mean

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accounted for by CO₂, 25% in protein, 2.5% in RNA, and 4.3% in lipids. More data must be obtained to confirm these preliminary results before abnormal situations can be effectively investigated.

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B. Methods of procedure.

The experiments in the early part of the investigation will be done first with rat lungs and then with dog lungs. The basic experiments, as we now conceive them, are described below. The experimental design and direction will depend, however, on the results of preceding experiments.

1. Confirmation of preliminary data and investigation of substrate utilization.

Initially, we intend to elucidate and confirm our early findings (see "Background") by means of the standard Warburg manometric technique (1,2). Rat lung slices are cut free-hand by standard techniques and placed in 5-ml Warburg vessels with buffered physiologic solution at pH 7.4 and an osmolarity of 280 to 300 at 37°C. The center well contains 0.7 ml of 20% potassium hydroxide for CO₂ trapping with the filter paper in place to increase the area of absorption of CO₂. Various radioactive and nonradioactive substrates are added (see below), and incubations are carried out for suitable time periods. Currently, we are using 30, 60, 90, and 120 minutes. The reaction is stopped with perchloric acid, and the indicated analyses are performed. The data are expressed per milligram of nitrogen or wet weight. Nitrogen analysis will be performed by the standard micro Kjeldahl techniques (3). The necessary control experiments will be run concurrently with liver. All anaerobic experiments will be carried out with 100% nitrogen.

2. Evaluation of the glycerophosphate shuttle.

Because of the depressed activity of the α -glycerophosphate enzyme in the cytosol of neoplastic tissue, which impairs the shuttle system of cancer cells, we feel that an initial approach would be to see if the same sort of mechanism was present in normal pulmonary tissue. Utilizing standard techniques, the cytosol of lung tissue will be collected and the α -glycerophosphate dehydrogenase activity will be measured fluorometrically (4). Utilizing tissue slices, and later isolated lung cells prepared by the method of Gould (5), the tissue levels of α -glycerophosphate and dihydroxyacetone will be evaluated utilizing fluorometric techniques (4). Although we are aware of the problems involved and the objections to the use of tissue homogenates, perhaps some of these experiments, for technical reasons, will be performed with this type of preparation.

The significance of the α -glycerophosphate pathway as an NADH shuttle mechanism between cytoplasmic and mitochondrial spaces in lung cells will be studied by experimentally manipulating the tissue level of α -glycerophosphate with the use of inhibitors. First, amino-oxyacetate will be added to the perfusion period in order to inhibit the flux of reducing equivalents via the malate-aspartate shuttle mechanisms (6). Lactate or glucose will be used as substrate, and aliquots of the perfusion fluid and freeze-clamped tissue will be analyzed for metabolic intermediates by fluorometric methods (4). Analysis of the effect of the inhibition on the rate of aerobic glycolysis and lactate oxidation will give

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Information concerning the activity of the α -glycerophosphate shuttle (6). Lungs will also be perfused with iodoacetamide to inhibit the activity of glyceraldehyde-3-phosphate dehydrogenase. Tissue levels of α -glycerophosphate and dihydroxyacetone phosphate will be measured to determine α -glycerophosphate shuttle activity. These experiments with inhibitors will also be done with glycerol and xylitol as substrates to determine if α -glycerophosphate can be produced in lung tissue via glycerol kinase and via the hexomonophosphate shunt. Because α -glycerophosphate is thought to be a key intermediate in phospholipid production (7), knowledge of the potential metabolic sources of this compound is important. If the glycerophosphate shuttle appears to be absent, an evaluation will be attempted of the malate-aspartate shuttle.

3. Measurement of glucose uptake aerobically and anaerobically.

For the measurement of glucose uptake, rat lung slices are incubated with ^{14}C uniformly labeled glucose under the appropriate condition in the Warburg apparatus. At suitable time intervals the reaction is quenched with 7% perchloric acid, and the change in glucose concentration is measured either by enzymatic techniques (8-10) or by the method used in I. A. Rose's laboratory using column chromatography. The neutralized solution is put on a Dowex-1 acetate column with some carrier glucose, lactate, etc. Because the glucose is uncharged, it will pass right through, while the Krebs cycle acids, phosphate esters, etc., will stick. The glucose therefore elutes in the region of the breakthrough peak. The acidic components are then eluted using an HCl gradient, and by the use of cold carriers the fraction can be analyzed and counted. By this method we hope to be able to quantitate the distribution of the ^{14}C and measure the glucose uptake. Lactic acid production will be measured by standard enzymatic techniques (11), and oxygen utilization and CO_2 production will be measured by standard manometric techniques (1).

4. Determination of the rate of production or utilization and the origin of lactic acid aerobically and anaerobically.

It is important to measure the rate as well as the origin of lactic acid production anaerobically and aerobically in the presence and absence of a substrate. For substrate we intend to use initially glucose, a carbohydrate, and a 4% rat serum albumin containing bound palmitate or octanoic acid as a representative lipid. Oxygen utilization, CO_2 production, and lactic acid production will also be determined. Comparison of the C-1/C-6 ratio of CO_2 production both anaerobically and aerobically, utilizing C-1 and C-6 labeled glucose permits an estimate to be made of the contribution of the pentose shunt. CO_2 radioactivity will be measured along with oxygen utilization and lactic acid production.

5. Determination of the precursor origin of phospholipid.

We have shown that lactic acid is produced in significant amounts. Our initial experiments indicate that the lung can utilize none of this lactate as a substrate, but this must be confirmed by further investigation. Utilizing uniformly labeled ^{14}C -acetate as the substrate both anaerobically and aerobically, measurements of radioactive CO_2 , oxygen consumption and fractionation into acid soluble fractions, lipids, proteins, RNA, DNA, etc., will be performed (12). Subsequent determination of radioactivity will indicate the distribution of substrate byproducts. It is possible that the glucose and the palmitate moieties

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give rise to different parts of the phospholipids made by the lung. Synthesis of phospholipids and surfactants appear to be important biosynthetic functions of the lung (13). The results of Felts (14) suggest the possibility that the ^{14}C from glucose tends to go predominantly into the glycerol fragment. This suggested compartmentalization between the carbohydrate and lipid utilization pathways might involve some very interesting regulatory aspects. Without "hard" numerical data, it is pointless to speculate on the possibilities.

Double labeling experiments under aerobic and anaerobic conditions will be performed in which both glucose and acetate or fatty acid are labeled, the former with ^{14}C and the latter with tritium. Following quenching in the usual manner with perchloric acid and neutralization, the lipids will be extracted with acetone:ether:chloroform (2:2:1), and the lipid fraction will be counted for ^{14}C and ^3H .

Hydrolysis in boiling 1M NaOH should lead to hydrolysis of the lipid into glycerol and fatty acid and inorganic phosphate (from phospholipid). Ether extraction under alkaline conditions will extract the glycerol and leave the fatty acid in the alkaline solution. Counting the glycerol for ^3H and ^{14}C radioactivity should indicate its origin. The alkaline solution is then acidified, rendering the fatty acids insoluble, and they can be extracted with chloroform and then counted for the double radioactivity. The residual acidified aqueous solution should be counted also as a check for incomplete extractions. Analysis of the distribution of label will indicate the origin of the various components of the lipid fraction.

6. Determination of the effects of CO_2 and O_2 on substrate utilization and biosynthesis.

Two groups of experiments are contemplated. The first deals with the utilization of approximately 5.6% CO_2 in the atmosphere during incubation, since this is the approximate concentration of CO_2 in the alveolar gas. It is of interest to investigate whether this elevated CO_2 is of any biosynthetic importance. In the second group of experiments PO_2 will be regulated, utilizing concentrations such as 100%, 70%, 50%, and 30% oxygen in order to determine if the PO_2 has any regulatory effect on the substrate utilization.

7. Evaluation of isolated mitochondrial regulation.

Because of the regulatory importance of the Krebs cycle enzymes and intermediates, we plan early investigations in this area, which will aid in the interpretation of the data of other experiments. The special enzymatic mechanisms by which the Krebs cycle intermediates can be regulated and replenished are called anaperotic reactions. Recent research has shown that the pyruvate dehydrogenase system is also inhibited by ATP, which is therefore a negative modulator. Whenever the ATP level in the cell exceeds a certain point, the pyruvate dehydrogenase system, which provides fuel for the tricarboxylic acid cycle, "turns off." In addition, it has been shown that the NAD-linked isocitrate dehydrogenase is the normal catalyst for isocitrate oxidation in the tricarboxylic cycle; the NADP-linked enzyme is primarily concerned with auxiliary biosynthetic reactions of the cycle (3,16).

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In a series of experiments, mitochondria of rat or rabbit lungs will be harvested by techniques of differential centrifugation. Oxygen uptake with various substrates, particularly fatty acids as substrates (palmitate) will be measured with a polarograph. Utilization of these substrates will be determined, and the effects of metabolic inhibitors will be studied. Either glycolysis or fatty acid oxidation may be responsible for providing the CoA for these mitochondria. The fact that lactate is noted to increase in quantity would indicate the probability that pyruvate is not the prime source of CoA. The energy produced can be utilized either for ATP production or for ion transport. Measurements of ATP, ATPase and ion transport (particularly Ca^{++}) will be performed utilizing the procedures outlined by Mela and Chance (16). If palmitate appears to be an important substrate, the possible requirement of carnitine will be investigated. We will compare the in vitro results with those obtained in the study of other tissues, including liver, and their mitochondria. This information will enable us to characterize oxygen and substrate characteristics of the lung. The basic techniques have been well utilized in the study of liver by J. R. Williamson of this Institution. We plan to use his techniques of fluorometric assay, which are described in his numerous papers and summarized in Methods of Enzymology (4). From his experiments Dr. Williamson was able to define certain regulatory mechanisms involved in liver synthesis and metabolism, and the utilization of these techniques should lead to very useful information regarding pulmonary metabolism. These techniques will allow for the evaluation of Acetyl CoA as well as pyridine nucleotide oxidation-reduction ratios, which will enable us to evaluate the total energy potential of the system. Such information should enable us to proceed to a more detailed investigation of individual reactions.

8. Isolated, perfused lung preparation.

Perfused whole lungs appear to be superior to tissue slices for the study of lung metabolism (17). Lungs will be removed from fasted rabbits, ventilated by a piston pump with gas containing 5% CO_2 in air, and perfused in a pulsatile manner via the pulmonary artery and veins with Krebs bicarbonate buffer solution containing 6% serum albumin. A closed system with a venous reservoir will be used for the perfusion. The lungs will be weighed, and the total volume of the perfusate will be measured by dilution of Evans Blue. The preparation will be immersed in Krebs solution equilibrated with CO_2 in air. Various substrates will be investigated, including glucose, lactate, amino acids, and selected fatty acids. Aliquots of the perfusion mixture will be taken at 30-minute intervals and analyzed for glucose, lactate, and pyruvate. Biopsies of the lung will be taken by rapid freezing between tongs precooled in liquid N_2 , and the tissue will be analyzed by fluorometric methods (4) for pyruvate/lactate, α -glycerophosphate/dihydroxyacetone phosphate, β -OH butyrate/acetoacetate and NADH/NAD levels to determine redox state (18) and ATP will be measured to evaluate energy supply. Similar experiments will be performed with the addition of lactate instead of glucose to the perfusate. In some experiments, ^{14}C -1 or ^{14}C -6 labeled glucose will be added to the perfusion medium; $^{14}\text{CO}_2$ will be recovered by passing the expired air through a CO_2 absorber, and the trapped radioactivity will be measured by scintillation counting. The ratio between CO_2 produced from C_1 and C_6 labeled glucose will be used as an index of hexosemonophosphate shunt activity.

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The significance of the α -glycerophosphate pathway as an NADH shuttle mechanism between cytoplasmic and mitochondrial spaces in lung cells will be studied by experimentally manipulating the tissue level of α -glycerophosphate, as described in section 2.

We will also use the isolated, perfused lung to study pathways of free fatty acid metabolism. As noted previously, there is considerable discrepancy in the literature about the ability of lung to oxidize fatty acids. ^{14}C -labeled palmitate and octanoate bound to albumin (19) will be added to the perfusion mixture, and the $^{14}\text{CO}_2$ evolved will be measured. The rate of change of free fatty acids in the perfusate will be measured spectrophotometrically (20). Further studies will depend on these initial results. For example, if palmitate oxidation is impaired, a palmitylcarnitine transferase inhibitor, (+) decanoylcarnitine, will be added to test for the requirement of carnitine for palmitate oxidation (21), and carnitine tissue levels will be measured.

We will further study oxidative metabolism of the lung by applying the technique of surface fluorometry, using a spinning disc fluorometer (22). For these experiments, the lungs will be insufflated with air or oxygen, and ventilation will be suspended. The fluorescence probe will be applied to the lung surface while perfusion continues. We will follow the fluorescence changes as the perfusion medium is altered with respect to substrate concentration and composition, and this will be compared to fluorescence changes produced by ventilating the lung with N_2 . With this technique we can simultaneously monitor fluorescence changes due to pyridine nucleotides and, with an absorption channel, measure changes due to flavoproteins. In this way we can pick up the respiratory chain at several sites to determine the effect of substrate in a physiological setting on intracellular redox state.

9. Comparison of animal data with human data.

After we have acquired an adequate amount of information about the pathways and regulation of metabolism from our animal studies, we will carry out comparative investigations of normal human pulmonary tissue. Tissues will be obtained from operative specimens taken from patients undergoing surgical procedures for known medical indications.

10. Individual cell components.

At future stages in this project, such cells of the pulmonary parenchyma as can be isolated by standard techniques, such as alveolar macrophages, will be individually investigated for their role in the overall metabolic processes, which we will understand better at that time.

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11. Biosynthetic pathways.

In future stages of the program, studies of biosynthetic pathways and their regulation will be carried out for surface-active substances and vasoactive substances (including prostaglandin) which appear to be either synthesized or degraded by the lung. Techniques described above with both isolated, perfused lung and lung slices will be utilized. Confirmatory in vivo experiments will be carried out when feasible.

12. Evaluation of the effects of smoke upon pulmonary metabolism.

Utilizing techniques similar to those described above, the effects upon normal pulmonary metabolism of tobacco smoke will be determined. In one group of experiments in vitro evaluation will be determined in closed vessels containing

either smoke or some of its components. In in vivo experiments animals will be placed in plexiglass chambers in which the atmospheric conditions of O_2 and CO_2 and the percent of smoke can be controlled. The exposure time will vary from short-term (a few hours or days) to long-term (one to four weeks). Physiologic measurements of pulmonary function will be carried out where feasible. At the end of this period the animals will be sacrificed, and either lung slices or perfusion techniques will be utilized for the evaluation of metabolism. In particular, we plan to investigate the Ca^{++} and Mn^{++} transport of mitochondria. These ions have been shown to be affected by local anesthetics (23). It is to be noted that in this investigation no attempt will be made to design new methods of instrumentation or technology. We will use well-established experimental techniques in an effort to gain new information and insights into pulmonary metabolism.

References:

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2. Kleinzeller, A. Manometrische Methoden. Verlag, Prague, 1964.
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4. Williamson, J.R., and Corkey, B.E. In, Methods in Enzymology, Vol. 13, J.M. Lowenstein, ed. Academic Press, New York, 1969, p. 434.
5. Gould, K.G., Jr., et al. *Science* 178:1209, 1972.
6. Safer, B., Smith, C.M., and Williamson, J.R. *J. Mol. Cell. Cardiol.* 2:111, 1971.
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9. Hill, J.D., and Kessler, E. *J. Lab. Clin. Med.* 57:970, 1961.
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11. Boehringer and Sons, Analytical Kits.
12. Fleck, A., and Munro, H.N. *Biochim. Biophys. Acta* 55:571, 1962.
13. Said, S., Klein, R.M., Norrell, L.W., and Maddox, Y.T. *Science* 152:657, 1966.
14. Felts, J.M. *Med. Thorac.* 22:89, 1965.
15. Atkinson, D.E. *Ann. Rev. Biochem.* 35:85, 1966.
16. Mela, L., and Chance, B. *Biochemistry* 7:4059, 1968.
17. O'Neill, J.J., and Tierney, D.F. *Fed. Proc.* 30:697, 1971.
18. Veech, R.L., Eggleston, L.V., and Krebs, H.A. *Biochem. J.* 115:609, 1969.
19. Milstein, S.W., and Driscoll, L.H. *J. Biol. Chem.* 234:19, 1959.
20. Novak, M. *J. Lipid Res.* 6:431, 1965.
21. Nasr, K., and Heinmann, H.O. *Amer. J. Physiol.* 208:118, 1965.
22. Scholz, R., et al. *J. Biol. Chem.* 244:2317, 1969.
23. Mela, L. *Arch. Biochem. Biophys.* 123:286, 1968.

1003540168

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The research facilities of the Department of Surgery and the Department of Physiology of the University of Pennsylvania School of Medicine will be available for these studies. (The principal investigator has a joint appointment in these departments.) There are animal laboratory facilities at both the Graduate Hospital and the Harrison Department of Surgical Research of the University of Pennsylvania. The major pieces of equipment needed for radioisotopic determinations and biochemical determinations are available to us. If needed in the future, larger animals and animals of other species are readily obtainable through the facilities of the School of Veterinary Medicine of the University of Pennsylvania. In addition, Dr. Leena Mela, a disciple of Dr. Britton Chance, has made her laboratory facilities available for collaborative studies.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Herbert W. Wallace, M.D.

Leena M. Mela, M.D.

T. Peter Stein, Ph.D.

Ellen M. Liquori, M.S.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
Attached.

1003540169

14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)

Herbert W. Wallace, M.D.

Principal
Investigator

40%

\$18,075

T. Peter Stein, Ph.D.

Biochemist

50%

9,038

Leena M. Mela, M.D.

Consultant

5%

0

Technical

Ellen M. Liquori, M.S.

Research
Technician

100%

12,075

Sub-Total for A 39,188

B. Consumable supplies (by major categories)

Rats

\$1,200

Rat care @ \$0.04/day

200

Chemicals, glassware, tubing

2,500

Radioisotopes

2,000

Sub-Total for B 5,900

C. Other expenses (itemize)

Travel (domestic)

600

Publications

400

Sub-Total for C 1,000Running Total of A + B + C 46,088

D. Permanent equipment (itemize)

Fluorometer

6,000

Recorder

1,200

Justification: A fluorometer and
a recorder are not available to us
and will have to be purchased and
modified for the purposes described
in this proposal.

Sub-Total for D 7,200E 6,913Total request \$ 60,201

E. Indirect costs (15% of A+B+C) * 5% annual increase

15. Estimated future requirements: **Upkeep & repair of equipment

***Includes cost of buying, boarding and operating on dogs as well as rats

Salaries * Consumable Suppl. ***Other Expenses Permanent Equip. *** Indirect Costs Total

| | Salaries * | Consumable Suppl. *** | Other Expenses | Permanent Equip. *** | Indirect Costs | Total |
|--------|------------|-----------------------|----------------|----------------------|----------------|--------|
| Year 2 | 41,147 | 7,500 | 1,000 | 500 | 7,447 | 57,594 |
| Year 3 | 43,204 | 7,500 | 1,000 | 500 | 7,756 | 59,960 |

1003540170

5.

16. Other sources of financial support: NONE

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| | | | |
| | | | |
| | | | |
| | | | |

PENDING OR PLANNED NONE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| | | | |
| | | | |
| | | | |
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for checks

Principal investigator

Typed Name Herbert W. WallaceSignature Herbert W. Wallace, M.D. Date 1/25/74Telephone 215 KI 6-5151 --
Area Code Number Extension

Responsible officer of institution

Typed Name _____

Title _____

Signature W. H. Wallace Date _____Telephone _____
Area Code Number Extension

1003540171

#958 - WEBB

1003540172

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1974

Grant application No. 958

CHRONIC PULMONARY DISEASE

To: The committee comprising Drs. Gardner, Liebow and Wyatt .

Subject: D. Robert Webb, M.D., Virginia Mason Research Center, Seattle
New application No. 958
"Proteolysis in Chronic Obstructive Lung Disease"

History

This proposal was case #249. As the Executive Committee requested additional information, the best course seemed to be to allow a full application.

Request

Application #958 requests \$27,372 plus two additional years.

Documents Submitted (attached)

1. Application dated January 7, 1974 (5 pages plus inserts).
2. Biographical sketches and bibliographies of Drs. Webb, Carroll and Morgan.

Comment

This proposal appears to be essentially a duplicate of one pending with the National Heart and Lung Institute.

FWN:gh

Encls.

FWN
F.W.N.

1003540173

956

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

JAN 29 1974

Date: Jan. 7, 1974

1. Principal Investigator (give title and degrees):

Dean Robert Webb, Jr., M.D., Associate Physician, The Mason Clinic

Co-Investigators:

Carrol J. Martin, M.D.,

Edward H. Morgan, M.D. Chief, Section of Respiratory Diseases, Dept.

2. Institution & address: of Internal Medicine, The Mason Clinic

Virginia Mason Research Center
1000 Seneca Street
Seattle, Washington 98101

3. Department(s) where research will be done or collaboration provided:

Department of Medicine, Virginia Mason Medical Center
Seattle, Washington 98101

4. Short title of study:

Proteolysis in Chronic Obstructive Lung Disease

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: Three years

7. Brief description of specific research aims: The overall objective of this project is to study the etiologic importance of protease-antiprotease balance in patients with chronic obstructive lung disease (COLD). Data reviewed in the appendix suggest this balance may be important in both human disease and in experimental models. Documentation of the importance of proteases and their control would open new avenues for the early detection, prevention, and perhaps therapy in patients with this clinical picture.

Three years are planned for this overall project. The natural history and pulmonary function of patients to be studied are being sequentially evaluated in another ongoing study. The specific aims for the first year are detailed below and include development of methods and the accomplishment of a small pilot study. The main study involving five groups of 20 patients each is planned for the second year. The patient groups to be studied are: I - normal controls, II - severe COLD, III - early COLD, IV - Chronic bronchitis, and V - bronchial asthma. After analyzing results from the main study in the third year, an additional restricted study will be performed on new patients to further document the validity of those findings.

Specific aims for the first year are as follows: (1) Study serum, secretions and leukocytes from 10 patients with carefully documented, severe COLD and ten control patients for differences in proteolytic capacity and anti-proteolytic activity. (2) Finish method development

1003540174

1-A

to accurately quantitate alpha₁ antitrypsin, immunoglobulin A, free secretory component, lactoferrin, and lysozyme in sputum and saliva. (3) Develop norms for the enzymatic potential of polymorphonuclear leukocytes in normals and COLD patients. (4) Use information gained from the initial pilot study to finish planning details of the main study.

1003540175

8. Brief statement of working hypothesis:

Many data both in humans and experimental animals are consistent with the hypothesis that emphysema, which presents clinically as COLD, results from uncontrolled proteolysis in the lung.

In this hypothesis the normal milieu, with proteases appropriately controlled by protease inhibitors, does not result in disease. However, either deficiency of inhibitors (in alpha, antitrypsin deficiency, for example) or excessive protease (probably present in chronic bronchitis) may destroy this balance and result in lung destruction. The rationale of this study is to measure several variables which might lead to increased protease activity and/or decreased inhibitor activity in patients with COLD and control patients without disease. The goal is documenting whether protease-antiprotease balance is altered in patients with COLD. A population has been selected for study which is being comprehensively evaluated sequentially, thus insuring that all aspects of history and physiologic studies will be at hand to document any lung disease present without significant cost to this project. Many of the proposed studies on serum and secretions are already being performed so their issue in this study is relatively assured.

9. Details of experimental design and procedures (append extra pages as necessary)

Definitions: For the purpose of this study chronic obstructive lung disease (COLD) will mean a syndrome characterized by obstruction on forced expiration which is not responsive to bronchodilators. These patients may be symptomatic with cough, dyspnea, and sputum, or asymptomatic. Patients with atopic history of episodic bronchospasm in early life, eczema, hay fever, or recurrent urticaria will be excluded from this category. Chronic bronchitis will be defined consistent with the British Medical Research Council criteria as history of chronic sputum production for at least three months in the last two successive years. Bronchial asthma is defined for this study as recurrent episodic bronchospasm in a patient with normal pulmonary function after bronchodilator therapy and with two of the following four criteria for the atopic state - positive skin tests to common antigens, a family history of atopy, a personal history of eczema, and a history of seasonal allergic rhinitis.

Patient Population and its Selection. The patients used for this study will come from a larger study of the psychological aspects of COLD supported by the National Heart and Lung Institute under the Pulmonary Specialized Centers of Research Program (HL 14152 M2). These patients have the results of a detailed history, physical examination and physiologic evaluations stored for easy retrieval by computer. Pulmonary functional studies include lung volumes, forced expiratory spiograms before and after bronchodilators, maximal voluntary ventilation, nitrogen washout studies, membrane permeance by carbon monoxide steady state technique, arterial blood gases at rest and during exercise, dynamic and static compliance, closing volumes and oscillometric pulmonary resistance. These patients are being followed on a yearly basis with the array of physiologic studies above. As of September 1973, 250 patients have been entered into the study and have had initial evaluation; 75 patients have had follow-up physiologic evaluation one year after entry.

Selection of patients for each of two groups in the pilot study and five groups in the main study will be by perusal of the results of the P-SCOR

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9. Procedures continued. (1)

evaluations. All patients will be between 45 and 75 years old and have alpha₁ antitrypsin phenotype MM by acid starch gel electrophoresis. Qualifications for each group are listed below.

Group I. Normal Controls - No history of chronic respiratory symptoms. Normal pulmonary function within accepted norms used at this institution.

Group II. Advanced COLD - History of chronic pulmonary symptoms. Physiologic screening studies will show both peak expiratory flow rate (PEFR) and maximal voluntary ventilation (MVV) less than 40 percent of predicted. The mean 1 second forced expiratory volume (FEV₁) in this group is 39 percent of the total FEV.

Group III. Early but definite COLD - Pulmonary symptoms may or may not be present. Screening PEFR and MVV are between 70 and 85 percent of predicted. The mean FEV₁ for the group is 65 percent of total FEV.

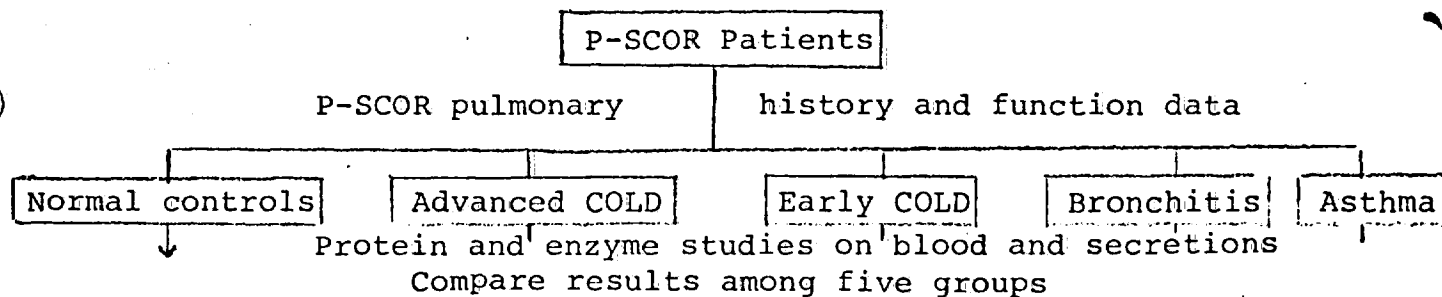
Group IV. Chronic Bronchitis - Essentially these patients have chronic sputum production and normal pulmonary function as described in Definitions.

Group V. Bronchial Asthma - This group is described in Definitions. As described below under Potential Problems (Appendix), they serve as a source of sputum from patients with essentially no destructive lung disease. This sputum will probably not be available from normal controls.

Three separate phases are necessary to accomplish the goals of this research proposal. Each phase can be conveniently regarded as taking one year. Phase I entails final development of methodology for various measurements and performance at the pilot study using ten patients with advanced COLD and 10 control patients. Phase II will be the main study of 100 patients to be evaluated during the second year in the groupings listed above. Phase III involves the final analysis of data from the main study and the accomplishment of a smaller restricted study to further document validity of results of the main study. Further details of these three phases can be seen in the Appendix under Tentative Work Schedule.

Grouping of patients in the main study (second year) for comparisons of blood and secretion studies is shown diagrammatically in the Flow Sheet below. In the initial year only control and severe COLD patients will be studied.

PATIENT FLOW SHEET FOR PROTEOLYSIS STUDY



1003540177

9. Procedures, continued. (2)

Historical and Physiologic Data. Selected data will be extracted from the P-SCOR study for correlations with results of studies on blood and secretions done for this project. Historical points to be recorded will include age, sex, smoking history, sputum production, exercise tolerance, duration of symptoms, and history of atopy. Physiologic results extracted will include lung volumes, FEV_1/FEV , maximal voluntary ventilation, membrane permeance, closing volumes, airway resistance, and compliance. The above data will be placed on computer cards for later analysis and correlation.

Studies on Blood and Secretions. Informed consent (see Appendix pg. A-12) will be obtained on each patient for obtaining a 40 cc blood sample, and for retaining a sample of sputum and saliva. These are the only studies that will be done on participants and follow-up evaluation for this project is not presently planned.

Collection and Storage of Samples. Each patient seen in the P-SCOR study will donate a sputum sample of 30 cc volume at the time of the physiologic studies. The 40 cc blood sample will be obtained at the time of physiologic studies also and the 30 cc saliva sample will be obtained from the large quantity usually stimulated at the time of the esophageal balloon studies of compliance. The leukocytes will be separated from blood as described below and frozen in several aliquots with the sputum and saliva samples at -70° centigrade for further analyses so each sample will have to be subjected to only one freeze-thaw cycle.

Blood studies will include those on leukocyte lysosomes and on serum. Lysosomes will be initially harvested by two methods: first, the method of Chodiker, Bock, and Vaughn^{37*} will be used to obtain lysosomes for chemical analysis of elastase-like esterase and neutral protease activity. Elastase-like esterase in the lysosomal fraction will be assayed by its action on synthetic substrates considered specific for elastase (tertiary butyloxy-carbonyl L-alanine p-nitrophenol and/or N-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester) as described by Janoff³⁸. Neutral protease will be measured in the lysosomal fraction by the method of Porter and Cebra.³⁹ Secondly, Dr. Inge Olsson of Lund, Sweden has agreed to collaborate in the study with specific immunological measurements of lysosomal proteases in his laboratories in Lund. He has isolated the two major proteases from polymorphonuclear leukocytes with great purity⁴⁰ and thus can measure these concentrations using immunologic means. One protein is an elastase and is inhibited by α_1 antitrypsin. The other is a collagenase-like material and is inhibited by α_2 macroglobulin. His measurements will be from lysosomal enzymes extracted from granulocytes after a Ficoll-Hypaque separation.

Serum studies will include quantitation of immunoglobulins by radial immunodiffusion and α_1 antitrypsin quantitation by electroimmuno-diffusion using a modification of the method of Laurell.⁴¹ Acid starch

*References are located in the Appendix after Background Material.

1003540178

9. continued (3)

gel electrophoresis with subsequent crossed electrophoresis as described by Fagerhol⁵ will be done to obtain AAT phenotype on each serum. Patients with other phenotypes than MM will be excluded to rule out AAT deficiency as a cause of COLD.

Sputum will be cultured for bacteria and a differential cell count will be made. AAT, Sec IgA, Secretory Piece, Lactoferrin, and Lysozyme will be quantitated by methods developed or under development in this laboratory based on the basic methods described independently by Laurell,⁴⁰ and Merrill, Harley and Claman.⁴² Attempts will be made to assay elastolytic and proteolytic activity of sputum directly by a modification of the methods described above under studies on the leukocytes. Specific enzymatic activity and specific protein quantitation will be evaluated in comparison to total protein concentration, dry weight, and to volume of an immediately preceding 24-hour sputum sample.

Saliva will have the same protein quantitations as sputum. This procedure is done with attention to the possible screening use of saliva as a much easier sample to obtain than sputum in most patients.

Analysis and Interpretation of the Data. A large number of individual observations will make analysis difficult and multivariate analysis will probably be necessary. Initial simple comparisons of the means between groups for each quantitation described above in blood and secretion studies will be made. Biostatistical help will be available for multivariate analyses. A major use of the initial pilot study will be used to provide experience with which to plan the data collection and analysis of the main study in more detail. A Raytheon 704 computer is used for P-SCOR data and is available to this study. The importance of early continuing interaction with biostatistical specialists is recognized and planned.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Virginia Mason Research Center is an independent research facility with laboratories, administrative staff, experimental surgery suite, and animal facilities to support multidisciplinary research. Active studies are currently underway in various aspects of respiratory disease including mechanisms of pulmonary fibrosis, physiologic studies of obstructive lung disease in both animals and man, collagen and elastin studies in obstructive disease, cell culture studies of various cell populations of the lung, laryngeal pathology, and physiologic changes caused by hyperbaric conditions.

Clinical resources are those of The Mason Clinic, a multi-specialty group practice of 85 physicians. Four Chest medicine and two Allergy specialists see large numbers of patients with COLD. Various clinical and research conferences in pulmonary disease and an active postgraduate training program promote interest in new developments in pulmonary disease. This interest in turn engenders excellent cooperation among staff members for research studies.

All equipment needed for the present is available except for a refrigerator-freezer which will be needed for storage of samples, and an electrophoresis power source. Other equipment available includes equipment for protein studies, electron and immunofluorescent microscopy, and a fairly complete cell culture laboratory. Equipment available and specifically needed for this study include tissue homogenizer, sonicator, agarose and starch gel electrophoresis apparatus, drying oven, and a high-speed centrifuge.

~~11. - Additional facilities required:~~

To summarize, the Virginia Mason Medical Center has ideal facilities with which to support the planned research project. Particular emphasis on pulmonary disease is present in both the clinical and research areas which gives a "critical mass" of interest and constructive criticism. Laboratory space and major equipment needed for this study is all on hand.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003540180

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

D. Robert Webb, M.D.

20%

\$6,000

fringe

1,200

C. J. Martin, M. D.

5%

-0-

Edward H. Morgan, M.D.

5%

-0-

Technical

Dick Chen

100%

\$8,000

fringe

1,040

Secretary (to be recruited)

25%

1,750

fringe

228

Sub-Total for A

\$18,218

B. Consumable supplies (by major categories)

Buchler Model #3-1155 Power Supply

685

S-8223 Vortex Genie Mixer (SCI prod)

65

4812 Cole Parmier Magnetic Stirrer & Hot Plate

114

Glassware

1,000

Antibodies

500

Chemicals

800

Clerical Supplies, Copying, etc.

200

Sub-Total for B

\$ 3,364

C. Other expenses (itemize)

Data Processing

300

Biostatistical Consultation

500

1 National Trip

500

Blood Counts & Sputum Analysis 20 Pts.

300

Air Freight (3 trips, Copenhagen @ \$28 + ea)

85

Sub-Total for C

\$ 1,685

Running Total of A + B + C

\$23,267

D. Permanent equipment (itemize)

Silver King 7 Cu Ft. Refrigerator

300

Silver King 7 Cu Ft. Freezer

315

Sub-Total for D

\$ 615

E

3,490.05

E. Indirect costs (15% of A+B+C)

Total request

\$ 27,372.05

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 23,990 | 3,000 | 3,000 | -- | 4,498 | 34,488 |
| Year 3 | 20,277 | 1,000 | 1,800 | -- | 3,461 | 26,538 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

| CURRENTLY ACTIVE | | | |
|---|--|----------|---------------------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| Protein Abnormalities in Obstructive Lung Disease | #172 Virginia Mason Research Center | \$9,814. | Nov 1, 1972 - Jan. 31, 1974 |
| Protein Abnormalities in Obstructive Lung Disease | #172 Virginia Mason Research Center | \$9,203 | Feb. 1, 1974 - Jan. 31, 1975 |

| PENDING OR PLANNED | | | |
|---------------------|--------------------------------------|----------|---------------------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| Proteolysis in COLD | National Institutes of Health (NHLI) | \$75,184 | May 1, 1974 - April 30, 1977 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Virginia Mason Research Center

Mailing address for checks

1000 Seneca Street

Seattle, Washington 98101

Principal investigator

Typed Name D. Robert Webb, M.D.Signature D. Robert Webb, M.D. Date 1/5/74Telephone (206) 623-3700 Ext. 344
Area Code Number Extension

Responsible officer of institution

Typed Name William F. Willoughby, M.D., Ph.D.Title DirectorSignature William F. Willoughby, M.D. Date 1/5/74Telephone (206) 624-1144 Ext. 417
Area Code Number Extension

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#901M - WEINBAUM

February 12, 1974

Grant application No. 901M .

CHRONIC PULMONARY DISEASES

To: The committee comprising Drs. Liebow, Meier and Wyatt

Subject: George Weinbaum, Ph.D., Albert Einstein Medical Ctr., Philadelphia
Modified application No. 901M
"Lung Proteinase: Antiproteinase Balance and the Effect of Cigarette
Smoke on this Interaction"

History

Application No. 901 in the amount of \$45,332 plus two additional years, was considered at the March 1973 SAB meeting and deferred for a site visit. Unfortunately the visit was not arranged until after the October 1973 SAB meeting.

Request

Application No. 901M requests \$38,163 plus two additional years.

Documents Submitted (Enclosed)

1. Letter from Dr. Weinbaum dated January 24, 1974.
2. Modified application, 1/16/74

Comment

To refresh recollections, we enclose copies of reports from Dr. Wyatt on a site visit conducted December 4, 1973; and from Dr. Liebow on a site visit conducted December 10, 1973.

FWN:gh
Enclosures

FWN
F.W.N.

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ALBERT
EINSTEIN
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CENTER

NORTHERN DIVISION
YORK and TANKER HOS.
PHILADELPHIA, PA 19141
(215) 329-0700

PHILIP KIMBEL, M.D.
Head
Pulmonary Disease Section

R. SINHA, M.D., Ph.D.

January 24, 1974

Frederic W. Nordsiek, Ph.D.
Associate Research Director
The Council For Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, N.Y. 10022

Re: Application #901

Dear Dr. Nordsiek:

We are enclosing two copies of our revised and updated application as requested by you on December 17, 1973.

We have included a section on recent progress in our laboratory as it pertains to our proteinase-antiproteinase project (p.3-6). We have also removed any studies concerned with species other than dog and human, in accord with suggestions by Drs. Liebow and Wyatt. Their questions and recommendations aided us greatly in tightening our research approach and making it more relevant to the human situation.

The project as we are submitting it to you is totally independent of any work that we are doing for N.I.H. Their grant involves fractionation of cell proteinases involved in emphysema development. Therefore, Dr. Kimbel and I are requesting support from The Council for Tobacco Research for the total project as it is submitted. Along these lines, we have attempted to prepare as tight a budget as possible and have reduced our previously submitted request by \$7,000 a year.

Thank you again for your consideration of our application and we sincerely hope that our revisions meet with your approval.

Sincerely yours,

George Weinbaum
George Weinbaum, Ph.D.
Bioscientist
Pulmonary Disease Section

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#9C1M

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 East 59th Street
New York, N.Y. 10022
(212) 421-8885

JAN 29 1974

APPLICATION FOR RESEARCH GRANT

Submitted: 1/15/73

Revised: 1/16/74

1. CO-PRINCIPAL INVESTIGATORS

GEORGE WEINBAUM, PH.D., Bioscientist, Pulmonary Disease Section
PHILIP KIMBEL, M.D., Head, Pulmonary Disease Section

2. INSTITUTION & ADDRESS:

Albert Einstein Medical Center
Northern Division
York & Tabor Roads
Philadelphia, Pa. 19141

3. DEPARTMENT'S WHERE RESEARCH WILL BE DONE OR COLLABORATION PROVIDED:

Pulmonary Disease Section and
Research Laboratories
Albert Einstein Medical Center

4. SHORT TITLE OF STUDY:

LUNG PROTEINASE: ANTIPROTEINASE
BALANCE AND THE EFFECT OF CIGARETTE SMOKE ON THIS INTERACTION

5. PROPOSED STARTING DATE: May 1, 1974.

6. ESTIMATED TIME TO COMPLETE: 3 years.

7. BRIEF DESCRIPTION OF SPECIFIC RESEARCH AIMS:

The twin objectives of this investigation are 1) to identify and quantitate the primary factor responsible for protecting the lung against the action of autogenous proteinases which have been previously shown in this laboratory to produce experimental emphysema in dogs - and 2) to examine the role of cigarette smoke on this interaction. We shall isolate, purify, characterize and quantitate the normally occurring substance, found in lung tissue which inhibits the activity of specific proteolytic enzymes and determine if this lung antiproteinase found in dogs has its counterpart in the human lung. The production of antiproteinases and their ability to interact with enzymes capable of inducing experimental emphysema in animals will be studied and evaluated

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in normal animals and those exposed to cigarette smoke. In order to bring this study as close as possible to the human condition, we will use human material (i.e. polymorphonuclear leukocytes, alveolar macrophages and lungs) which will be available to us by collaboration with the Medical Examiners' Office, the Division of Laboratories at Albert Einstein and Jeannes Hospital.

8. BRIEF STATEMENT OF WORKING HYPOTHESIS:

Our working hypothesis is that emphysema may be induced by the proteolytic activity of specific enzymes from polymorphonuclear leukocytes and/or pulmonary macrophages. The release of these enzymes may be stimulated by various air-borne pollutants. These enzymes overwhelm the local defense mechanisms in the lung, including such factors as serum and tissue antiproteases, and destroy or alter the elastic tissue of the alveoli.

9. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES:

PART I - INTRODUCTORY STATEMENT

Epidemiologic studies suggest that smoking is an important factor in the development of pulmonary emphysema in humans. Two major, non-mutually exclusive theories for its induction differ only in their emphases on initial tissue attack. In the vascular theory (1), the initiating lesion is of vascular origin, resulting in obstruction of branches of the bronchial or pulmonary blood supply. The subsequent deficiency of nutrients would then lead to necrosis of alveolar walls and septa to form the characteristic emphysema pathology. According to the second theory (2), emphysema develops due to the direct attack of proteolytic enzymes at the air-lung interface. Regardless of which theory is correct there is eventual extensive damage to both the vascular and parenchymal tissue. A logical source of this degradation could conceivably be intracellular collagenase and/or elastase, released into the surrounding tissues due to cellular necrosis caused by such stimuli as chronic infection or chronic pollution-induced destruction.

Although cigarette smoking and human emphysema have been statistically related using clinical and autopsy material, it has not yet been shown if there is a direct causal relationship or if smoking and other pollutants merely accelerate a normal aging process. Also, if there is a direct relationship between smoking and emphysema it is still possible that smoking is but one of a number of extrinsic factors able to act synergistically with autogenous agents to effect this lung damage. Those experimental studies which have been performed on the effects of smoking do, however, suggest reasons for exploring the action of cigarette smoke as a single contributing factor in emphysema induction.

When human pulmonary leukocytes from smokers and non-smokers were compared (3, 4, 5), even asymptomatic smokers had greatly increased numbers of primarily polymorphonuclears and macrophages with a direct correlation to the

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amount of smoking and smoke inhaled. This cannot be explained as simply a protective effect, i.e., more phagocytes are supplied to remove an increased amount of foreign matter, because there was an actual decrease in the antibacterial activity of alveolar macrophages isolated from smokers. This effect differed among the various brands of cigarettes tested and varied inversely with the effectiveness of filtration (6). The significance of this decrease in antibacterial activity as opposed to such other findings as change in size or pigmentation, lies in the ability of the phagocytosed, undegraded particles to cause the lysis of the engulfing leukocyte with subsequent release of enzymes able to degrade pulmonary tissue.

The demonstrated greater susceptibility of cigarette smokers to respiratory infections may, therefore, be due to this combination of insufficient leukocyte degradation of invading organisms and the damage resulting from the action of liberated proteases on lung tissue (7). Since studies of human pulmonary emphysema are hampered by time required for induction and moral considerations, animal models have been employed. Although the horse has the advantage of having lung anatomy, distribution of bronchial arteries, and a natural emphysema similar to that found in humans, economy dictated that another animal model be used. Since this laboratory has successfully produced emphysema-like lesions in dogs (8), using aerosolized leukocyte proteinases and since the parenchymal effects of smoking in dogs resembled human emphysema (9), this system will be used as our initial model with the understanding that we will attempt to use human material when feasible in order to directly relate our observations to the human condition.

It has been suggested (10) that a decreased level of serum alpha₁-antitrypsin is of primary importance in correlating with the development of hereditary emphysema. Other investigators (7,11), however, have been unable to demonstrate a relationship between the development of emphysema and smoking in those individuals having intermediate or normal levels of this antiprotease. Although this may be a characteristic of the species studied, the report (12) that lung tissue binds the serum antiproteases suggests that not only is the total level of antiprotease important but also its localization and its availability to bind and thus inhibit proteolytic enzymes. Material from fractionated lung tissue must be examined for its role in proteinase-antiproteinase balance. Indeed, it is also possible that a lung specific antiproteinase may be present at the air-lung interface. Such antiproteinases may be of paramount importance as regulators of proteinases released from various cells in the lung during normal physiological states as well as during stress situations.

PART II - PREVIOUS WORK DONE ON THIS PROJECT

Since our original application was submitted we have much recent data in support of the concept of a lung antiproteinase acting as a primary defense system directed against those leukocyte enzymes capable of digesting lung tissue. Along these lines we have isolated, by lung lavage, an extracellular material which appears to have unique antiproteinase properties. The data are shown in Table I.

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Table 1

Comparison of Various Antiproteases
From Dog and Human Sources

| Enzyme | Substrate | Dog Lung Inhibitor | Maximum % Inhibition By | | | Human Lung Inhibitor |
|-----------------------------------|-----------|-----------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| | | | Dog Serum | Human α_1 -AT | Human α_2 -MG | |
| Purified Trypsin | BAPNA | 22 | 87 | 70 | 14 | -- |
| | Casein | 0 | -- | 68 | 43 | -- |
| Pancreatic Elastase | t-BOC-Ala | 91 | 50 | 95 | 30 | 0 |
| | Casein | 0 | -- | 0 | 22 | -- |
| Dog PMN Elastase | t-BOC-Ala | 93 | 70 | 100 | 22 | -- |
| Dog Alv. Macro- phage Elastase | t-BOC-Ala | 90 | 65 | -- | -- | -- |
| Human PMN Elastase | t-BOC-Ala | 65 | 30 | -- | -- | 80 |

The data presented in Table I shows that dog lung inhibitor does not inhibit trypsin to any great extent (22%) whereas dog serum or human α_1 -AT inhibit trypsin activity at least 70%. In addition, the lung antiprotease is a more effective inhibitor of pancreatic elastase than the dog serum antiproteases (91% vs. 50%). In this regard the dog lung inhibitor is markedly different than the human lung inhibitor, since the human lung antiprotease does not appear to inhibit pancreatic elastase. In obtaining our human lung material from the Medical Examiners' Office we have arranged to get normal specimens (gunshot deaths, suicides or auto accidents) when the autopsy is performed within 2 to 3 hours of death. Collection of such material has been approved by our Committee on Research Involving Human Subjects.

The experiments described above show that the dog lung antiprotease is different than the major dog or human serum antiproteases. The data for human lung inhibitor was obtained from a 1M NaCl extract of human whole lung homogenate (Janoff, personal communication) and the material is contaminated with lung tissue proteins to a far greater extent than our saline lavage. We must now determine if saline lavage of human lung allows isolation of such a unique lung antiprotease.

In addition, we have shown previously that polymorphonuclear leukocyte (PMN) homogenates will produce experimental emphysema in dogs and, more recently, have fractionated this PMN homogenate to isolate and characterize the emphysema-inducing agent. Acetone powders were prepared from dog PMN which were obtained by dextran sedimentation of fresh citrated dog blood. The desiccated material was extracted sequentially with water, 0.15M NaCl, 0.5M NaCl, 1.0M NaCl and 8M urea and the proteolytic activities of each of the fractions were determined using various substrates. General proteolysis against denatured hemoglobin was present in all the fractions. Elastolysis was found in the 1M NaCl and 8M urea fractions. Proteolysis using the residue remaining after 1M NaCl extraction of perfused, lavaged, dog lung homogenates as substrate was greatest in the 0.15M NaCl fraction, as is seen in Table II.

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Table II

Characteristics of Various Dog PMN Fractions

| <u>PMN Fraction</u> | <u>Emphysema-Inducing Activity</u> | <u>Lung Residue Digestion Activity</u> | <u>% Inhibition of Lung Residue Digestion Using Lung Antiproteinase.</u> | <u>Elastolytic Activity</u> |
|---------------------|------------------------------------|--|--|-----------------------------|
| H ₂ O | -- | 505 | 0 | -- |
| 0.15M NaCl | ++ | 1260 | 67 | -- |
| 0.5M NaCl | + | 924 | 64 | -- |
| 1.0M NaCl | -- | 253 | 0 | + |
| 8.0M Urea | -- | 840 | 0 | + |

As can be seen in Table II, only the fractions capable of inducing experimental emphysema in excised dog lobes (0.15M and 0.5M) were inhibited by lung antiproteinase in their ability to digest lung tissue. The three other fractions were not inhibited by lung antiproteinase. Further purification of the 0.15M NaCl fraction by precipitation with 55% (NH₄)₂SO₄ and fractionation on CM-Sephadex increased markedly the purification of the emphysema-inducing activity. Emphysema production was inhibited by incubation of the active 0.15M NaCl fraction with ovomucoid, a known proteinase inhibitor. The PMN fraction responsible for emphysema production is a protease which is able to degrade lung tissue but cannot be described as having classically assayable collagenolytic or elastolytic properties (Table II).

Furthermore, we have partially purified and partially characterized the lung antiproteinase. The supernatant was concentrated 100-fold by either lyophilization or ultrafiltration yielding a solution containing approximately 5 mg/ml of protein. After dialysis against 0.01M phosphate buffer, pH 7.5, the sample was fractionated on DEAE cellulose using a discontinuous salt gradient. Stepwise elution with 0.03M, 0.06M, 0.10M and 0.50M NaCl in the phosphate buffer yielded four peaks of protein with a recovery of 85-100%. The distribution of protein was approximately 25% in peak I, 18% in peak II, 14% in peak III, 36% in peak IV. Most of the antiproteinase activity was recovered in peak II, measured by inhibition of hydrolysis of t-BOC-alanyl-p-NO₂ phenyl ester by porcine pancreatic elastase. Acrylamide gel electrophoresis at pH 7.0 in 1% sodium dodecyl sulfate showed marked enrichment of a high molecular weight component in peak II as compared to the other three peaks.

Immunologic techniques demonstrated that there were differences between this lung antiproteinase and serum antiproteinase. Antisera were prepared against whole dog serum as well as against unfractionated lung antiproteinase. Crude antiproteinase reacted with both antisera in Ouchterlony plates. Using immunoelectrophoresis the crude antiproteinase showed at least three arcs with anti-dog serum and at least two arcs

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against antiserum to lung inhibitor. The partially purified antiproteinase of peak II showed a single arc against anti-dog serum. The arc was not coincident with that formed against partially purified dog alpha₁-antitrypsin.

This lung antiproteinase, while it showed immunologic cross-reactivity with serum proteins, was also differentiated from known dog serum antiproteinases on the basis of inhibition studies against two purified enzymes, trypsin and elastase. Under conditions where dog serum inhibited trypsin esterolysis maximally 90%, lung antiproteinase inhibited maximally 20%; whereas dog serum inhibited elastase esterolysis no more than 50% the lung antiproteinase inhibited more than 90%.

These data suggest that there may be a unique antiproteinase present at the air-lung interface. This inhibitor may play an important role in regulation of proteinases released into the lung under various physiological conditions.

With these data in mind, we propose the following research program.

PART III

A. RESEARCH PLAN OUTLINE

1. To Characterize Antiproteinase(s) in the Lung.
 - a. Isolate and characterize the factor(s) from dog and human lung responsible for antiproteinase activity.
 - b. Compare the factor(s) found in the dog and human lung with serum proteins demonstrating antiproteinase activity.
2. To Determine the Role of Smoking on the Interaction between the Proteinases and Antiproteinases in the Lung.
 - a. In vitro assay of proteinases from alveolar macrophages, lung tissue and PMN's and the qualitative and quantitative effects on them due to exposure to cigarette smoke.
 - b. In vitro assay of serum and lung antiproteinases from normal and smoking dogs.
 - c. Comparison of ease of induction of experimental emphysema using proteinases isolated from normal and smoking dogs on both types of dogs.

B. ABSTRACT OF RESEARCH APPROACH PROPOSED

1. To Characterize Antiproteinase(s) in the Lung
 - a. Isolate and characterize the factors from dog and human lung responsible for antiproteinase activity.
 - 1) use at least two different fractionation systems (homotenzation or lavage) to isolate dog and human lung antiproteinase

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2) quantitate the amount of antiproteinase normally present in dog or human lung

3) purify the antiproteinase by chromatographic (DEAE, CM and Sephadex) procedures

4) determine its molecular weight, if there is sugar or lipid associated with the protein and the mechanism of inhibition of specific proteinases

5) determine if the lung antiproteinases can prevent the development of enzyme-induced emphysema in vivo in the dog, and in vitro in isolated human lung lobes

b. Compare the factor(s) found in the dog and human lung with serum proteins demonstrating antiproteinase activity.

1) analyze the purified lung and serum antiproteinases by acrylamide gel electrophoresis and peptide map characterization

2) determine if the antiproteinase of the lung is immunologically related to any of the serum antiproteinases

3) compare the inhibition spectra of specific enzymes by the lung and serum antiproteinases

2. To Determine the Role of Smoking on the Interaction Between the Proteinases and Antiproteinases in the Lung.

a. In vitro assay of proteinases from alveolar macrophages, lung tissue and PMN's and the qualitative and quantitative effects on them due to exposure to cigarette smoke.

1) assay fractionated proteinases from alveolar macrophages and PMN of normal subjects and those exposed to various regimens of cigarette smoke

2) assay release of proteinases from alveolar macrophages or PMN before and after exposure to cigarette smoke

b. The effect of smoking on serum and lung antiproteinase levels and activities.

1) assay sera and lung lavage for changes in antiproteinase activities comparing normal and smoking subjects

2) determine if there has been a change in the relative amounts of each type of serum inhibitor after smoking

3) establish if there is any immunologic change in any of the antiproteinases after smoking

c. Effect of cigarette smoke on induction of proteinase-induced.

1) establish if smoking makes the lung more susceptible to leukocyte-induced emphysema.

2) determine if the alveolar macrophages or PMN from smoking subjects are equally effective as normal cells in inducing emphysema.

C. EXPERIMENTAL PROCEDURE

1. Characterization of Lung Antiproteinases

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Background

Human material will be studied not only as this is the species of primary concern, but also because no clearcut relationship has as yet been found between the intermediate levels of serum proteinase inhibitor and development of emphysema in smokers (11). One approach, that of genetic analysis of the numerous alleles involved in production of serum alpha₁-antitrypsin is being undertaken in several other laboratories (12). Our approach is a biochemical one in which we will evaluate the role of proteinase:antiproteinase balance under smoking and non-smoking conditions in relation to the development of experimental emphysema. Dog material will be employed due to the considerable body of data already available from this lab using this animal. Also, a direct relationship was found between macroscopic parenchymal disruption in dogs and duration of daily cigarette smoking over a period of 0-22 months (13). Microscopically, these lesions resembled human emphysema (13). Emphysema has been successfully produced in our laboratory in vivo using papain or leukocyte homogenate instillation or aerosolization. We have also developed an in vitro system for emphysema production utilizing isolated lobes and instillation of proteinases (14).

a. Isolation and Comparison of Dog and Human Lung Antiproteinases

Isolation and Quantitation of Tissue Inhibitors

Lung tissue from dog and human origin will be minced, homogenized in a blender, and washed three times with water followed by centrifugation at 15,000 rpm. The pellet will be washed twice, including once overnight, with 1M NaCl. The sodium chloride supernate will then be re-centrifuged at 42,000 rpm (15). Supernate extracts will be assayed for their ability to inhibit the proteolytic activity of enzymes extracted from polymorphonuclear leukocytes using the hemoglobin assay (16). It is expected that antiproteinase activity will be found in this fraction because of the preliminary work of Lieberman (17) and Janoff (18). We will initiate this work using the NaCl extract, but will check other lung extraction procedures also. As mentioned in Part II we have already initiated an extraction procedure utilizing lung lavage and have found that this isolation procedure yields an antiproteinase which may be different than the Janoff inhibitor (see Table I). However, since Janoff's work was done with human lung and ours with dog lung we will use both procedures on both species of lung tissue in order to determine which technique will allow for easy isolation in a relatively pure form and permit quantitation of antiproteinase levels (mg/gm wet lung tissue; mg/mg surfactant phosphorus or mg inhibitor protein/dog).

Characterization of Inhibitors

In order to characterize these antiproteinases they will be purified using such standard procedures as precipitation with ammonium sulfate, methanol, trichloroacetic or perchloric acid and various types of ion-exchange chromatography (19). An alternative procedure will be sought using the principle of affinity chromatography (20). Crystalline trypsin will be bound to Sepharose 4B using the cyanogen bromide method

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of Cuatrecasas and Anfinsen (21), and the matrix poured into a column. Lung extract or lavage containing the antiprotease will then be applied to the column, nonbound protein washed off, and the adhering inhibitor selectively eluted. As was described in Part II, we have already developed a chromatographic procedure to partially purify lung inhibitor using DEAE cellulose columns. We will characterize the purified inhibitor as to molecular weight (determined by Sephadex and acrylamide gel electrophoresis in sodium dodecyl sulfate as well as by ultracentrifuge analysis). We will also determine if it is a glycoprotein or a lipoprotein and if it has any chemical or immunological relationship to surfactant, which is also present at the air-lung interface. Finally, we will establish more clearly the interaction between the antiprotease and the enzymes listed in Tables I and II which it inhibits in order to understand the mechanism and requirements for inhibition.

Effect of Inhibitors on Induction of In Vitro Emphysema

In vitro emphysema has been induced in our laboratory using isolated lobes of dog lungs. Following removal of the lungs from the body the individual lobes were instilled with solutions of proteinase(s) and the enzyme(s) allowed to digest the tissues for 1½ hours at room temperature (14). We will determine if the inhibitors isolated above will effect in vitro induction. Formaldehyde will be instilled into the lungs at a standard pressure of 25cm. The organs will be immersed in formaldehyde for 48 hours, mounted, sliced and stained. Sections will be examined for alveolar wall destruction using the method of Dunhill (22). This procedure will allow us to better define what we are considering to be emphysema-like lesions and also to quantitate the effects of various doses of agents required to produce a defined level of lung destruction. The use of isolated lobes will provide us with a considerable economic advantage over in vivo work employing the entire animal. It will also allow us to minimize the effect of any animal to animal variation on a given experiment. We have preliminary data showing that lung antiproteinase is a strong inhibitor of emphysema development in vitro and we will expand these studies to determine if this inhibition works in vivo. We will also attempt to induce emphysema-like lesions in human lobes in vitro using human PMN enzyme and establish if there is a human lung antiproteinase which can regulate disease induction in a manner similar to the dog system.

b. The Relationship between Lung and Serum Proteolytic Inhibitors

Although both the serum and the pulmonary tissues have been reported to contain substances able to inhibit proteolytic enzymes no one has studied the possible relationship between the inhibitors from these two sources. The value of such a comparison lies in the relationship of these inhibitors to both the etiology and prognosis of the disease state, always with the assumption that since proteolytic enzymes are significant in these processes so too are these inhibitors. If a serum inhibitor is actually a subunit of a lung inhibitor (or vice versa) then it could be expected that there would be some sort of quantitative relationship between their concentrations. Conditions regulating this ratio could

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be involved in pathogenesis of pulmonary emphysema. Genetic abnormalities affecting the specific regulatory gene(s) would also be expected to affect both serum and pulmonary inhibitor if they were derived from the same molecule. Conversely, if the lung and serum antiproteases are not subunits of some primal molecule any relationship between the concentration of one as compared to the other could have some non-genetic, e.g., environmental origin.

Several methods listed below will be employed to study the relationship between antiproteases of the lung and the serum.

1. Polyacrylamide gel electrophoresis, with and without sodium dodecyl sulfate will be used to determine if the antiproteases isolated from dog or human lungs have subunits such as easily dissociable or reducible polypeptide chains and if they are similar to serum antiproteases isolated in a similar chromatography procedure.
2. Antisera will be raised in rabbits injected with purified serum antiprotease and tested against lung antiprotease using the immunoelectrophoretic and Ouchterlony techniques to find out whether or not the molecules share antigenic, and therefore, structural sites. As already described in Part II we have prepared antisera to whole dog serum, whole human serum, crude lung antiprotease and we are presently preparing antisera against purified dog α_1 -antitrypsin, α_2 -macroglobulin, partially purified lung antiprotease, as well as against purified human α_1 -antitrypsin and human α_2 -macroglobulins. The latter two substances were obtained through the generous help of the American Red Cross. We have already established that the partially purified lung antiprotease contains a component which reacts with antiserum developed against whole dog serum: that this component migrates in immunoelectrophoresis as an inter-alpha protein and that there are no other cross-reactive proteins present in the partially purified preparation. To determine if the cross-reactive protein is the lung inhibitor will require complete purification by procedures described in earlier sections. We will also carry out this immunologic analysis on the human lung lavage in order to bring this work closer to the human emphysema system.
3. Samples of each inhibitor will also be hydrolyzed and subjected to two-dimensional electrophoresis and chromatography, producing two-dimensional peptide maps in order to further compare the primary structure of the proteins.
4. Each inhibitor will be tested against such standard proteolytic enzymes as trypsin, collagenase, papain and elastase using hemoglobin and other pure proteins as substrates to obtain a general idea of its inhibitory spectrum.
5. Each inhibitor will also be tested for its effect on enzymes derived from dog or human polymorphonuclear leukocytes and macrophages, using as substrates material obtained from fractionated lung tissue to more closely approximate its actual effect in the intact animal. These experiments will expand the work already described in Tables I and II.

All these studies will aid us in understanding the nature of the dog and human lung antiproteases, their interaction with various cellular

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proteinases which may be present in the lung under certain physiological conditions and their relationships to dog and human serum antiproteinases. This work would have potential importance in understanding the etiology of emphysema, may aid in making earlier and more accurate diagnosis and may help to develop new techniques of treatment including the possibility of immunotherapy.

The people involved in the studies described above include Drs. George Weinbaum, Philip Kimbel and Bruce Sloan, as well as our biochemistry and histology technicians. The provisional timetable for accomplishing the work described above is approximately 24-30 months. Some of this work will run simultaneously with that described below.

2. The Role of Cigarette Smoke in Determining Proteinase:Anti-Proteinase Interaction

Background

The response of lung tissue to both acute and chronic treatments with cigarette smoke have been amply described micro- and macroscopically. Several laboratories (9,13) have supplied data detailing the numerous changes resulting from in vivo exposure of pulmonary tissue to noxious fumes but none has sought to determine the effect of such exposures on the interaction between autogenous proteinases and antiproteinases.

a. Effect of Smoking on Proteinases from Lung Macrophages, Lung Tissue, and Polymorphonuclear Leukocytes.

Populations of lung macrophages will be obtained by lavage (3). PMNs will be obtained from whole blood by the usual procedures used in our laboratory (23). Lung cells will be prepared by mincing lung tissue and homogenizing in a Potter-Elvehjem homogenizer, disrupted by sonication, and made into acetone powders. The powder will be extracted using water, 1M NaCl, and 8M urea to affect an initial fractionation corresponding roughly to acid, neutral, and alkaline proteinases. Work currently in progress in our laboratory using the PMN material from dog blood (as described in Part II) indicates: 1) the water extract is much richer in acidic proteinase (catheptic) activity than the combined neutral and alkaline activities; 2) the acidic activity is much less in the sodium chloride extract and essentially zero in the urea extract; 3) the sodium chloride extract has the greatest amount of alkaline and neutral activities; 4) the neutral and slightly acidic (pH 5) activities of the urea extract are more significant than either the acidic or alkaline proteolytic activities; 5) elastolytic activity, using t-Boc-L-alanine ester as substrate, is significant only in the high salt and urea extracts; 6) collagenase activity, using bovine achilles tendon as substrate was found in both the water and the low salt extracts, with minimal activity in higher salt or urea extracts. These findings are important in that they 1) demonstrate an initial significant separation of the major proteolytic activities and 2) the two principal enzymatic activities involved in connective tissue destruction, i.e., elastase and collagenase, and implicated in causing disease in these tissues (24) are easily separated. Although routine elastase assays are performed using a synthetic substrate, the use of elastin-orcein has confirmed the fact that the high salt and urea fractions did indeed have the elastolytic activities. As described in

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Part II we have succeeded in fractionating the dog PMN further to give us a partially purified enzyme fraction which contains less than 1% of the total cell protein and this fraction is the only one capable of inducing emphysema in isolated dog lobes. This work is being expanded to examine the purification of the emphysema-inducing agent from dog alveolar macrophages, human PMN and human alveolar macrophages utilizing, initially, the same purification scheme which has worked for the dog PMN enzyme.

The emphysema-inducing enzyme from dog PMN has been enriched in lung-digesting capacity. Therefore, those enzymes capable of destroying lung-derived substrates will be employed in the studies dealing with proteinase: antiproteinase interactions.

Macrophages will be washed from lungs of dogs exposed to cigarette smoke. The smoke exposure will be performed using a mask system as described in the letter submitted to Dr. F. Nordsiek on February 8, 1973, or we will use an updated system similar to that described by Battista and co-workers (25). We will rely on the experts recommended by the Tobacco Research Council to select the most satisfactory exposure system. Acute treatment will consist of short exposure to cigarette smoke adjusted to give an amount equivalent to two packs for a smoker of average weight. Chronic treatment will involve using cigarette smoke adjusted to give an amount equivalent to one-half pack in one group and two packs in a second group for varying intervals of time over a 0-6 month period.

The proteolytic activity of the macrophages will be determined using standard protein and synthetic substrates and also the specific lung substrates described above. Proteolytic activity will be measured not only using acetone powders of sonicated cells, but also from supernatants of cells allowed to merely discharge their enzymes due to possible changes in intracellular stability. These determinations of proteolytic activity will be performed on cells from both normal and treated dogs since there are reports (26) that smoking causes an increase in pulmonary proteolytic activity. Such data should be important in ascertaining if certain pollutants can potentiate the development of emphysema via the mechanism of proteolytic degradation.

b. The Effect of Smoking on Serum and Lung Anti-proteinase Levels and Activities.

Sera from dogs subjected to acute and chronic smoking schedules will be assayed for changes of antiproteolytic activities using both the antitryptic assay of Eriksson (27) and the more specific enzyme-substrate systems described above. Inhibition of specific antiproteolytic activities will be followed using the agar gel electrophoretic technique of Duchtler (28), in order to demonstrate not only different levels, but also different inhibiting spectra of antiproteolytic agents. The immunoelectrophoretic technique will tell us if there has been a shift in the amounts of α_1 -antitrypsin, α_2 -macroglobulins and inter-alpha antiproteinase components by utilizing the specific antisera presently being prepared. These data will, therefore, aid in determining if cigarette smoke affects the level or composition of the various serum anti-proteinases.

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Lung antiproteinase isolated as described in an earlier section will be assayed to determine possible changes due to exposure to cigarette smoke. The total amount of lung antiproteinase, the amount bound in the lung to proteinases and the ratio of these values will be determined to give a quantitative picture of the relationship of smoking to antiproteinase activity. Qualitative differences will be sought for by redetermining the spectra of enzymes inhibited by lung antiproteinase after exposure to cigarette smoke and comparing these to normal values.

c. Effect of Cigarette Smoke on Induction of Proteinase-Induced Emphysema.

Emphysema induction will be studied both in vivo and in vitro. Initially, our studies using PMN homogenates to produce emphysema will be extended to dogs previously subjected to various regimens of smoking as described in the preceding section. In our acute in vivo studies dogs exposed to cigarette smoke will be treated with various amounts and types of leukocyte-derived proteinase, sacrificed, and the severity of emphysematous lesions compared to those of untreated dogs. Chronic studies, as described previously, will also be performed prior to proteinase treatment to measure the short and long term effects of exposure to smoke on the ease of emphysema-induction using leukocyte proteinases.

In vitro studies will be carried out by removing lungs from dogs exposed to cigarette smoke and the isolated lobes used as test organs for proteinase studies. This method is not only more economical than whole animal studies but also emphasizes the effects of smoke and proteinases on the lungs themselves, with a minimum of extrapulmonary involvement due to circulatory transport of serum factors to act as antibodies, serum antitrypsin proteins or serum proteolytic enzymes. Previous work in the laboratory has demonstrated the feasibility of this technique for quantitating the amount of enzyme required to induce emphysema in the intact animal and should be of considerable utility in the program.

The significance of this program will lie in its determination of the role played by smoking on the development of emphysema. By studying the effect of cigarette smoke on both the serum and lung antiproteinases we hope to demonstrate which antiproteinase is more important in conditions likely to cause emphysema. We will also better understand the role of proteinase-antiproteinase balance during induction of experimental emphysema. We hope to utilize the observations made in our model system in understanding the sequence of events occurring during the development of human pulmonary emphysema. In accomplishing this we believe that we shall be better able to describe those individuals most prone to emphysema development and, eventually, suggest a method of treatment. The smoking experiments described in this section will only be performed on dog materials. The people involved are the same as listed in the previous specific aim (pg.10). This work will be initiated 6-12 months after characterization of lung antiproteinases has been begun and our suggested timetable is that this work will take 24-30 months to accomplish.

1003540198

C. REFERENCES

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2. Strawbridge, H., Amer. J. Pathol. 37, 161 (1960).
3. Harris, J.O., Swenson, E.W. and Johnson, J.E., J. Clin. Invest. 49, 2086 (1970).
4. Lellouch, J. and Schwartz, D., Lancet 2, 632 (1971).
5. Pratt, S.A., Finley, T.N. and Smith, M.H., Anat. Record. 163, 497 (1969).
6. Green, G. and Carolin, D., New Eng. J. Med. 276, 421 (1967).
7. Flint, G., Maxwell K. and Renzetti, A., Arch. Environ. Health, 22, 366 (1971).
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9. Haynes, W., Kristulovic, V., and Bell, A., Amer. Rev. Resp. Dis. 93, 730 (1966).
10. Eriksson, S., Acta Med. Scand. 175, 197 (1964).
11. Fudenberg, H. and Larson, R., p.65 in Pulmonary Emphysema and Proteolysis. Ed. by C. Mittman, Academic Press (1972).
12. Liebermann, J., P. 189 in C. Mittman. op. cit.
13. Hernandez, J.A., Anderson, A.E., Holmes, W.L. and Foraker, A.G. Amer. Rev. Resp. Dis. 93, 78 (1966).
14. Weinbaum, G., Marco, V., Ikeda, T., Mass, B., Meranze, D.R. and Kimbel, P., Amer. Rev. Resp. Dis. (in press, 1974).
15. Work in progress.
16. Anson, M.L., J. Gen. Physiol. 20, 565 (1937).
17. Lieberman, J., Trimmer, B.M. and Kurnick, N.B., Lab. Investig. 14, 249 (1965).
18. Janoff, A. and Scherer, J., J. Exptl. Med. 128, 1137 (1968).
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20. Cuatrecasas, P. and Anfinsen, C.B., p.345 in Vol. XXII, Methods in Enzymology, ed. W.B. Jacoby, Acad. Pr. (1971).

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22. Dunhill, M.S., Massarella, G.R. and Anderson, J.A., Thorax 24, 176 (1969).
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24. Johansson, W.G., Pierce, A.K. and Southern, P.M., Amer. Rev. Resp. Dis., 103, 908 (abstract) 1971).
25. Battista, S.P., Guerin, M.R., Gori, G.B. and Kensler, C.J., Arch. Environ. Health, 27, 376 (1973).
26. Lunan, K. and Freeman, G., p.463 in C. Mittman, op. cit.
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28. Duchterlony, O., Handbook of Immunodiffusion and Immunelectrophoresis. Ann Arbor Science Publ. (1968).

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10. SPACE AND FACILITIES AVAILABLE:

The facilities available in the Biochemistry Laboratory for use during these investigations include:

1. Zeiss phase microscope
2. Sorvall centrifuge
3. Hitachi-Perkin Elmer spectrophotometer
4. Mettler balance
5. Refrigerator and freezer
6. Glassware for all the basic techniques described

The facilities available in the Cardiopulmonary Laboratories for use during these investigations include:

1. Eight-channel FM magnetic tape recorder with voice input.
2. Six-channel FM magnetic tape recorder with voice input.
3. Filtering and differentiator circuit.
4. Differential and vascular pressure transducers.
5. Animal body plethysmograph (modified small body respirator).
6. Spirometers.
7. Gas chromatograph for CO and N₂ analysis.
8. Godart CO Analyzer.
9. Godart CO₂ Analyzer.
10. Beckman O₂ Analyzer.
11. Blood gas and pH electrodes system with water bath and tonometer.
12. Pressure cycled respirator (Bird Mark VII).

The Research Laboratories have the following general facilities available:

1. Hotpack walk-in incubator
2. Walk-in cold room
3. RCA electron microscope
4. Dark room facilities

Animal boarding facilities occupy an adjoining building. An animal surgical suite is located there, contains a completely equipped operating facility and is maintained by a full-time staff. Standardization of dogs is practiced and during a three-week period of observation, testing and treatment prior to experimentation, pre-existing medical problems are eliminated.

11. ADDITIONAL FACILITIES REQUIRED:

NONE

1003540201

- 17 -

12. BIOGRAPHICAL SKETCHESA. GEORGE WEINBAUM, Ph.D.

Bioscientist, Pulmonary Disease Section

Born: **REDACTED**Role in Project: Co-Principal InvestigatorEducation, Training, Honors

University of Pennsylvania, Philadelphia, Pa. - A.B.

The Penna. State University, University Park,
Penna. - M.S.The Penna. State University, University Park,
Penna. - Ph.D.

Tokyo U. Inst. Appl. Micro, Japan - Postdoctoral

Albert Einstein Medical Center, Philadelphia, Pa.

Post-doctoral

1961

Fulbright Research Scholar, Tokyo University

1959-60

Career Development Awardee, National Inst. of Gen.
Med. Sciences

1969-74

Professional Experience

I am presently an Associate Member in the Biochemistry Department. My research at Albert Einstein Medical Center has involved studies on animal and bacterial cell membrane structure and synthesis, enzyme biosynthesis and regulation, biosynthesis of naturally occurring nucleoside analogs in fungi, abnormal cell wall synthesis and characterization of the lipids of E. coli cell wall complexes. I am a Career Development Awardee.

From 1957-61, I was director of the Biochemical Section of the Pathology Department at Geisinger Medical Center, Danville, Penna. My research involved amino acid analogs and tissue culture cells. I spent one year (1959-60) as a Fulbright Research Scholar at the Institute of Applied Microbiology in Tokyo University. I was studying exoenzyme synthesis in B. subtilis.

I received my Ph.D. from Penna. State Univ. in 1957, having worked with Dr. M. F. Malleite on induced enzyme synthesis in E. coli.

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- 18 -

B. PAULIP KIMBEL, M.D.

Head, Pulmonary Disease Section

Born: **REDACTED**Role in Project: Co-Principal InvestigatorEducation, Training, Honors

Temple University (Major in Sciences)

Temple University School of Medicine - M.D.

Internship, Albert Einstein Medical Center

Residency, Internal Medicine, Albert Einstein Medical Center

USPHS Post-Doctoral Research Fellowship

Department of Physiology and Pharmacology

Graduate School of Medicine

University of Pennsylvania

Alpha Omega Alpha Honor Medical Society - Award for highest examination scores, senior medical class

Diplomate, American Board of Internal Medicine

Professional Experience

Head, Pulmonary Disease Section, Albert Einstein Medical Center, Philadelphia, Pennsylvania

1961-

Professor of Medicine, Temple University Health Sciences Center School of Medicine, Philadelphia, Pennsylvania

1971-

Associate Member, Research Laboratories, Albert Einstein Medical Center, Philadelphia, Penna.

1968-

Associate Professor of Medicine, Temple University Health Sciences Center School of Medicine

1957-71

Associate in Medicine, Temple University Health Sciences Center School of Medicine

1963-67

Instructor in Medicine, Temple University Health Sciences Center School of Medicine

1960-63

Research Associate, Fels Research Institute, Temple University School of Medicine and Institute for Cancer Research (with Dr. S. Weinhouse - Blood Glucose Metabolism). Part-time

1958-61

Research Associate, Department of Physiology, Graduate School of Medicine, University of Pennsylvania (part-time). Worked with Dr. A.B. Dubois and Dr. H. Linderholm in studies of Pulmonary Capillary Blood Flow Simultaneously with Cardiac Catheterization

1958-61

REDACTED

1003540203

- 19 -

C. BRUCE SLOAN, Ph.D.

Research Associate

Born: **REDACTED**Role in Project: Research AssociateEducation, Training, Honors

Temple University - B.A.

Hahnemann Medical College - M.S.

Hahnemann Medical College - Ph.D.

Department of Pathology, Harvard Medical School
Post-doctoralDepartment of Microbiology, Albert Einstein Medical
Center - Post-doctoral

Temple University - Dean's list

Hahnemann Medical College, U.S.P.H.S. Predoctoral
FellowProfessional Experience

I am presently a postdoctoral fellow in the Biochemistry Department, Pulmonary Disease Section, at Albert Einstein Medical Center. My research here has consisted of studies on proteolytic enzymes derived from dog polymorphonuclear leukocytes and macrophages. I have been attempting to assay, isolate and define the role of enzymes on the development of experimental emphysema using the dog as a model system.

From 1969-1971, I was a postdoctoral fellow in the Laboratory of Chemical Pathology, Department of Pathology, Harvard Medical School. My research was concerned with the role of genetics and the state of the antigen on cellular and humoral immune mechanisms.

I received my graduate training at Hahnemann Medical College, under Dr. Peter Stelos. My research was concerned with studies of the structure of immunoglobulin G, specific antibodies, and Bence-Jones proteins and employed enzymatic and chemical procedures for protein degradation and sequencing.

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13. PUBLICATIONS:

A. G. Weinbaum, Ph.D.

Okuda, S. and Weinbaum, G., Immunologic Cross-Reactivity of Escherichia coli B, Envelope Glycoproteins with Some Animal and Plant Cell Membrane Proteins, J. Immunol., 103:869 (1969).

Weinbaum, G., Fischman, D.A. and Okuda, S., Membrane Modifications in Nutritionally Induced Filamentous Escherichia coli B, J. Cell Biol. 45:493 (1970).

Marco, V., Mass, B., Meranze, D.R., Weinbaum, G. and Kimbel, P., Induction of Experimental Emphysema in Dogs Using Leukocyte Homogenates, American Review of Respiratory Disease, 104:595 (1971).

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema, American Review of Respiratory Disease, 106:384 (1972).

Kimbel, P., Mass, B., Ikeda, T. and Weinbaum, G.: Emphysema in Dogs Induced by Leukocyte Contents, Pulmonary Emphysema and Proteolysis, p.411. Edited by C. Mittman, Academic Press, Inc., New York (1972).

B. P. Kimbel, M.D.

Kaplan, A.S. and Kimbel, P.: Pulmonary Capillary Blood Flow Waves in Subjects with Abnormal Pulmonary Hemodynamics, Journal of Applied Physiology, 28:793 (1970).

Marco, V., Mass, B., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema in Dogs Using Leukocyte Homogenates, American Review of Respiratory Disease, 104:595 (1971).

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema, American Review of Respiratory Disease, 106:384 (1972).

Kimbel, P., Mass, B., Ikeda, T. and Weinbaum, G.: Emphysema in Dogs Induced by Leukocyte Contents, Pulmonary Emphysema and Proteolysis, p.411. Edited by C. Mittman, Academic Press, Inc., New York (1972).

Marco, V., Meranze, D.R., Yoshida, M. and Kimbel, P.: Papain-induced Experimental Emphysema in the Dog, Journal of Applied Physiology, 33:293 (1972).

C. B. Sloan, Ph.D.

Sloan, E. The Extension of Thin Layer Electrophoresis on Cellulose to the Identification of DNS-amino acids. J. Chromatography 42:426 (1969).

Sloan, B. and Gill, T.J. Genetic and Cellular Factors in the Immune Response. IV. The Effect of Aggregation on Antibody Formation and on Delayed Hypersensitivity in the Inbred ACI and F344 Strains of Rats. Journal of Immunology, 108:26 (1972).

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13. PUBLICATIONS (continued)

Sloan, B. and Gill, T.J. Synthetic Polypeptide Metabolism. IV. In Vivo and In Vitro Degradation of Poly (Glu⁵² Lys³³ Tyr¹⁵) in Highly Responding (AC1) and Poorly Responding (F344) Strains of Rats. Immunochemistry, 9:677 (1972).

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14. FIRST YEAR BUDGET:

| A. <u>Salaries</u> | <u>% Time</u> | <u>Amount</u> |
|---|---------------|-----------------|
| <u>Professional</u> | | |
| George Weinbaum, Ph.D., Co-Principal Investigator | 20 | |
| Philip Kimbel, M.D., Co-Principal Investigator | 20 | |
| Bruce Sloan, Ph.D., Research Associate | 50 | |
| <u>Technical</u> | | |
| Biochemistry Technician | 100 | |
| Histology Technician | 50 | |
| Fringe Benefits | | |
| <u>Sub-Total for A</u> | | |
| <u>B. Consumable Supplies</u> | | |
| Animals | | 2,250 |
| Boarding of animals | | 750 |
| Glassware | | 500 |
| Chemicals, Drugs, Stains, etc. | | 1,000 |
| <u>Sub-Total for B</u> | | \$ 4,500 |
| <u>C. Other Expenses</u> | | |
| Travel to National Meetings | | 500 |
| Publication costs | | 250 |
| <u>Sub-Total for C</u> | | \$ 750 |
| <u>Running Total of A + B + C</u> | | \$32,750 |
| <u>D. Permanent Equipment</u> | | |
| 2 Animal Smoking Masks @ \$250 each | | 500 |
| <u>Sub-Total for D</u> | | \$ 500 |
| <u>E. Indirect Costs (15% of A + B + C)</u> | | 4,913 |
| <u>TOTAL REQUEST</u> | | <u>\$38,163</u> |

15. ESTIMATED FUTURE REQUIREMENTS:

| | <u>Salaries</u> | <u>Consumable Supplies</u> | <u>Other Expenses</u> | <u>Permanent Equipment</u> | <u>Indirect Costs</u> | <u>Total</u> |
|---------------|-----------------|----------------------------|-----------------------|----------------------------|-----------------------|--------------|
| <u>Year 2</u> | | \$4,500 | \$750 | - 0 - | \$5,288 | \$40,538 |
| <u>Year 3</u> | | \$4,500 | \$750 | - 0 - | \$5,588 | \$42,838 |

REDACTED

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16. OTHER SOURCES OF FINANCIAL SUPPORT:

CURRENTLY ACTIVE

| <u>Title of Project</u> | <u>Source</u> | <u>Amount</u> | <u>Inclusive Dates</u> |
|--|--------------------------|---------------|------------------------|
| <u>Etiology of Experimental Emphysema.</u> | N.I.H. - HE 13787-01 | \$140,000 | 5/1/71 to 4/30/74 |
| <u>Structure, Function and Synthesis of Cell Membranes</u> | N.I.H. - K04-GM-07259-04 | \$ 23,000 | 7/1/69 to 6/30/74 |

PENDING OR PLANNED

| <u>Title of Project</u> | <u>Source</u> | <u>Amount</u> | <u>Inclusive Dates</u> |
|-------------------------|----------------------|---------------|------------------------|
| <u>Renewal</u> | N.I.H. - HE 13787-04 | \$150,000 | 5/1/74 to 4/30/77 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to:

Mr. Jerome Baron, Vice-President for
Fiscal Affairs

Mailing address for checks:

Albert Einstein Medical Center - N.D.
York & Tabor Roads, Phila., Pa. 19141

Principal Investigators:

George Weinbaum, Ph.D.
Philip Kimbel, M.D.

Signatures:

George Weinbaum Date 1/22/74
Philip Kimbel Date 1/22/74

Responsible officer of institution

Mr. Steven Sieverts
General Director

Signature:

Steven Sieverts Date 1/25/74
Telephone: 215 DA 9-0700 Ext. 381

1003540208

CARDIOVASCULAR

1003540209

31013 - BING

1003540210

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 21, 1974

Grant application No. 31OR13

CARDIOVASCULAR

To: The committee comprising Drs. Jacobson, Liebow and Sommers

Subject: Richard J. Bing, Huntington Memorial Hospital, Pasadena
Application No. 31OR13
"Mechanisms of the Action of Carbon Monoxide on Atherosclerosis"

History

CTR has supported this investigator since 1955. His current level of support is \$39,893 a year.

Request

Application #31OR13 requests \$45,293. Dr. Bing states the increase is due to "...increasing cost of supplies as well as the inflationary spiral concerning salaries."

Dr. Bing's current grant was made "...without any assurance of further support beyond June 30, 1974." Nevertheless the long tradition is maintained of retaining his low number and consecutive numbering of repeated renewals.

Documents Submitted (attached)

1-Application dated 1/17/74.

2-Progress report #4, June 30, 1973 - July 1, 1974.

Comment

Reprints or manuscripts of the numerous recent publications listed by Dr. Bing are available. These have been, or will be sent to you as part of the regular ongoing distribution to SAB members.

FWN:gh

Encls.

FWN
F.W.N.

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act.
1/15/74
jh

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 58TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 18 1974

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☐

Date: 1/17/74

1. Principal Investigator (give title and degrees): Richard J. Bing, M.D.
Professor of Medicine, University of Southern California
Director of Cardiology and Intramural Medicine,
Huntington Memorial Hospital
Visiting Associate in Biomedical Engineering,
California Institute of Technology
2. Institution & address:

Huntington Memorial Hospital
100 Congress Street
Pasadena, California 91105

3. Department(s) where research will be done or collaboration provided:

Huntington Institute of Applied Medical Research
Huntington Memorial Hospital

4. Short title of study:

Mechanisms of the Action of Carbon Monoxide on Atherosclerosis

5. Proposed renewal date: July 1, 1974

6. How results to date have changed earlier specific research aims:

This is a continuation of the work sponsored during the last year.
New elements introduced concern the study of the mechanism of the
effect of carbon monoxide.

7. How results to date have changed earlier working hypothesis:

The effect of carbon monoxide on cholesterol uptake by human coronary
arteries has now been clearly established. The proposed work is based
on these findings.

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8. Any additional facilities now required? Describe briefly:

None.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

The biochemical execution of this work is now under the supervision of Dr. J.S. Sarma, whose curriculum vitae is enclosed. The other key personnel will remain the same with the exception of Dr. S. Ikeda who replaced Dr. J.M. Fauvel. The overall direction of this project remains with Dr. Richard Bing, the principal investigator.

10. Append outline of experimental protocol for ensuing year. see enclosed.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent). see enclosed.

12. Summary progress report (append in standard form as separate document, unless recently submitted). see enclosed.

1003540213

3.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)J.S. Sarma, Ph.D.
Fringe Benefits

90%

H. Tillmanns, M.D.
S. Ikeda, M.D.
R. J. Bing, M.D.

30%

30%

70%

REDACTED

Technical

A. Grenier, M. S.
Fringe Benefits

100%

K. Seeler, M. S.
H. Hansen, B. A.

70%

30%

REDACTED

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Isotopes, reagents, enzymes, catheters, syringes,
perfusion pump repair, general chemicals

\$10,500

pkay
gh

Sub-Total for B

\$10,500

C. Other expenses (itemize)

Expenses in obtaining arteries and veins and for
histological examination

2,000

Sub-Total for C

2,000

Running Total of A + B + C

\$38,690

D. Permanent equipment (itemize)

Lindbergh Pump

800

Sub-Total for D

800

E. Indirect costs (15% of A+B+C)

E

5,803

Total request

\$45,293

1003540214

14. Other sources of financial support.

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--------------------------------------|--|----------|--------------------|
| "The Effect of Alcohol on the Heart" | National Institutes of Health, AA00304-02 | \$38,700 | 1/1/72 to 4/30/74 |
| "The Coronary Microcirculation" | Margaret W. and Herbert Hoover, Jr. Foundation | \$34,000 | 3/1/73 to 2/28/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--------------------------------------|---|----------|--------------------|
| "The Effect of Alcohol on the Heart" | National Institutes of Health, 5 R01 AA00304-02 | \$58,868 | 5/1/74 to 4/30/77 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Checks payable to

Huntington Memorial Hospital

Mailing address for check:

Huntington Memorial Hospital
100 Congress St., Pasadena, California 91105

Principal investigator

Typed Name Richard J. Bing, M.D.Signature Richard J. Bing Date Jan 16, 74

Telephone (213) 796-0371 526
Area Code Number Extension

Responsible officer of institution

Typed Name Mel S. OrtmannTitle Business ManagerSignature Mel S. Ortmann Date Jan 16, 74

Telephone (213) 796-0371 510
Area Code Number Extension

1003540215

CURRICULUM VITAE

J. S. M. Sarma, Ph.D.

PERSONAL

Age: R

Birthplace: R

REDACTED

EDUCATION

B. S.c., Andhra University, Waltair, India

M. S., Andhra University, Waltair, India

Ph.D., Andhra University, Waltair, India

Research Associate, Wayne State University (1969-1972)
under the direction of Dr. John P. Oliver, department of
Chemistry.

Presently in charge of biochemical and chemical cardiac
research at the Huntington Institute of Applied Medical Research; affiliated
with the California Institute of Technology.

HONORS

Graduated with honors from Andhra University, Waltair, India.
Received government of India Research Fellowship Award, August
1965 to July 1968.

PUBLICATIONS

1. Sarma, J.S.M.: Nuclear magnetic resonance spectrum of azulene: Calculation of chemical shifts by Johnson and Bovey's method. Ind. J. Pure & Appl. Phys., 4:365, 1966.
2. Sarma, J.S.M., Hashimoto, H., Tillmanns, H., Mao, J.C., Bing, R.J.: Lipid metabolism in perfused human nonatherosclerotic coronary arteries and saphenous veins. Atherosclerosis, Jan. 1974.
3. Tillmanns, H., Ikeda, S., Hansen, H., Sarma, J.S.M., Fauvel, J.M. Bing, R.J.: Microcirculation in the ventricle of the dog and turtle. Submitted to Circulation Research.
4. Sarma, J.S.M., Tillmanns, H., Ikeda, S., Bing, R.J.: The effect of carbon monoxide on lipid metabolism of human coronary arteries. Submitted to Atherosclerosis.

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Page 2

J.S.M. Sarma, Ph.D.

PUBLICATIONS CONTINUED

6. Sarma, J.S.M., Oliver, J.P.: Calorimetric and proton magnetic resonance studies on the complexes of 3,5-Lutidine with boron trihalides (in preparation).

1003540217

APPLICATION FOR RENEWAL OF RESEARCH GRANT

Richard J. Bing, M.D.
Huntington Memorial Hospital
100 Congress Street
Pasadena, California 91105

Project Title: The Mechanism of Increased Cholesterol Uptake
by Coronary Arteries (In Vitro Studies)

Background of Proposed Work

As described in our Progress Report, we have now established the fact that human coronary arteries do not synthesize cholesterol, but that cholesterol is transferred directly into the vascular wall from the blood or from the perfusion fluid. In similar studies using saphenous veins, we were able to show that these veins taken from humans during operations also take up cholesterol from the perfusate, the degree of uptake depending on the perfusion pressure. We found that nicotine fails to influence cholesterol uptake or lipid synthesis; finally we established the fact that carbon monoxide leads to a marked increase in cholesterol uptake of human coronary arteries beginning at a concentration of 5% carbon monoxide in the perfusion fluid. Apparently carbon monoxide does not interfere with lipid synthesis in the arterial wall. Our results were obtained on human coronary arteries, since it was discovered that there exist marked species differences as between blood vessels of different species.

The work on carbon monoxide was of particular interest since it demonstrated that carboxyhemoglobin increases the permeability of human coronary arteries to cholesterol. In this respect, CO acts similar to

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hypoxia, but its effect is more pronounced. Parving also studied the transvascular protein flux during carbon monoxide exposure and confirmed that the disappearance rate from blood of ^{131}I -albumin increased about 50% after three hours of exposure to 20-25% carboxyhemoglobin. Astrup demonstrated that hypoxia as well as carbon monoxide significantly increase the permeability of endothelial membranes. For example, rabbits, when exposed to carbon monoxide, develop arterial lesions resulting in a considerable accumulation of lipids. They also demonstrated that these animals often accumulated fluid with a high protein content, with a picture of subendothelial edema. Pauli found that carbon monoxide exposure (20-25% carboxyhemoglobin) led to a 50% increase in glomerular filtration rate.

The results as presented in our Progress Report and to be published in the Journal of Atherosclerosis Research agree in general with these animal studies, demonstrating that the permeability of vascular wall of human coronary arteries is altered by carbon monoxide. Although our experiments were conducted in vitro, we interpreted our finding of increased cholesterol uptake under the influence of carbon monoxide as indicating increased permeability of the arterial endothelial layer to cholesterol flux. If one adheres to the concept that cholesterol is actively transported in the vascular wall, our findings imply that biochemical factors controlling the permeability of the arterial wall may be altered by CO. On the other hand, many studies suggest that lipid transport across the arterial wall is a purely passive physico-chemical process. If this is the case, then it could be assumed that CO

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modifies those factors which result in an increase in endothelial permeability. As we mentioned in our Progress Report and our publications, no difference in cholesterol uptake was found using two different levels (5 and 90%) of carbon monoxide in the perfusion fluid. This suggests that the rate of cholesterol uptake under these experimental conditions is an all or none process. Furthermore, no influence of carbon monoxide on lipid synthesis in the arterial walls could be demonstrated in our studies.

It is the aim of this investigation to study the mechanism of increased cholesterol uptake under the influence of carbon monoxide using techniques developed in this laboratory. We feel that the principal advantages of our methods are: 1) the experiments are conducted in vitro, so that individual factors can be separate, 2) we can study the effects of various concentrations of carbon monoxide, 3) we can employ arteries from all species, including human coronary arteries; and 4) we can alter at will the hemodynamic conditions with which the vessels are perfused.

Proposed Work

The aim of our proposed investigation is a study of the mechanism of carbon monoxide on cholesterol transfer in perfused coronary arteries.

The study may be divided into two approaches: 1) physico-chemical mechanisms, and 2) the study of hemodynamic causes of increased cholesterol transfer. As indicated in the previous paragraphs, describing the background of our work, carbon monoxide does interfere with the transfer of cholesterol across the arterial wall of human coronary arteries. This

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occurs regardless of concentration of carbon monoxide in arterial blood, as long as minimal concentration of from 5-8% is present in the perfusate.

The questions to be examined are: a) Can increased permeability be artificially produced? These studies will be carried out using collagenase, an enzyme, which has been shown by Jaffe and his associates (Journal of Clinical Investigation 52:2745, 1973) to lead to digestion of portions of the vascular wall, the degree of change depending on the time to which these vessels are exposed to the enzyme. Thus, for example, it was shown that in specimens examined after collagenase treatment, the endothelial cell lining was electively lost, leaving the basement membrane and underlying structures intact. If, however, collagenase was used in conjunction with manipulation of the vascular wall, the basement membrane was also destroyed.

In a recent series of experiments, the effect of hypoxia on the penetration of cholesterol in the vascular wall will be tested. As mentioned above, there are reasons to believe that the effects of carbon monoxide are at least in part due to hypoxia. For example, Ayres and associates (Archives of Environmental Health 26:8, 1973), demonstrated that the effects of carbon monoxide are primarily related to the leftward shift of the oxygen hemoglobin curve and perhaps also to combinations of carbon monoxide with myoglobin and certain iron-containing enzymes. Hemoglobin-oxygen equilibria in the presence of carboxyhemoglobin resemble the equilibria of more primitive forms of hemoglobin. The oxygen capacity of the blood is decreased, and there

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occurs an increase in coronary blood flow and in cardiac output. Britton Chance and Williams demonstrated that a change in respiratory rate produced an alteration in the redox state of the respiratory chain. The relative availability of oxygen, hydrogen-containing substrate, inorganic phosphate and adenosine diphosphate determined whether the chain is more or less oxidized. Since the absorption spectra of respiratory enzymes vary with their redox state, the degree of oxidation may be estimated by spectrophotometric techniques. Therefore, it has been of some concern that carbon monoxide may interfere with the function of all three iron-containing respiratory pigments, hemoglobin, myoglobin and certain cytochromes. This would indicate that CO not only affects permeability of vascular walls, but also cellular respiration directly.

A parallel phase of this investigation will be concerned with the role of hemodynamic factors in the "uptake" or penetration of cholesterol in the arterial wall. This has also been found to be of importance in the mechanism of atherosclerosis.

There are a number of publications dealing with hemodynamic factors in atherogenesis. They are summarized in a paper by Gessner in Circulation Research 33:259, 1973. Several factors are mentioned here which could influence transfer of cholesterol from the blood or the perfusion fluid into the vascular wall. They are the Reynolds number, an actually dimensionless number, which is indicative of the ratio of inertial forces to viscous forces. The Reynolds number can be mathematically defined from the mean velocity averaged over the tube cross-section, the internal diameter of the tube, the density of the fluid, and the absolute viscosity

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of the fluid. For viscous flow within a tube, the flow motion near the wall is retarded and a boundary layer develops. In essence therefore we have to examine the laminar to turbulent transitional behavior in a pulsatile flow.

The second factor which may alter vascular permeability to cholesterol is pressure and flow related. It is known that atheromatous plaques appear in regions of low pressure because a suction action exerted on the surface endothelium eventually causes the layers to selectively separate from adjacent tissue. This factor can be easily tested in our in vitro preparations.

The third mechanism exerts itself through wall shear stress. This leads to erosion of the endothelium occurring at sites where the local wall shear stress is relatively high. The theory usually considers simultaneous mechanisms by which arterial wall cholesterol levels may be altered.

Therefore, there must be a series of events which lead to an influx of cholesterol from blood into the vascular wall. There is also the possibility of an opposite movement of cholesterol (from the vascular wall into the blood).

Method of Procedure

The in vitro system of perfusion of human and animal coronary arteries published in three reports from this laboratory will again be utilized; however, both human and dog arteries will be used in these experiments. The reason why we can utilize animal arteries, which synthesize cholesterol, is that one can determine in the control as

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well as in the experimental series the ratio of ^{14}C cholesterol/ ^3H cholesterol in arterial walls. By means of this ratio one can derive de novo synthesis of lipids in the arterial wall; ^3H cholesterol represents cholesterol moved directly from the perfusate into the arterial wall, ^{14}C uptake represents de novo synthesis. The arteries will be perfused in a modified Carrel-Lindbergh pump at pulsatile pressure. They are perfused at pressures of about 120/80 mmHg, in those experiments in which the effect of collagenase and hypoxia are tested.

In the experiments in which we measure the effect of hemodynamic factors, perfusion and flow rates can be changed at will. Diluted fresh blood, either dog or human blood will be used in the perfusate.

Sterile techniques will be used during the preparation of perfusion, which will be carried out over a period of four hours at 37° centigrade. The gas which drives the fluid through the artery and which will come into the equilibrium with the perfusion fluid consists of 5% CO_2 , 25% oxygen and 70% nitrogen. To the perfusion fluid will be added 2- ^{14}C sodium acetate and cholesterol, 1, 2- ^3H . Cholesterol will be added through sonication as described by us in previous reports. It was shown by us that tritium radioactivity is located primarily in the alpha-2-lipoprotein and in the β -lipoprotein fraction of the perfusion fluid. Lipids will be analyzed in the perfusion fluid prior to and following perfusion. Analyses will be carried out on the blood vessels and the extraction of the lipids will be carried out according to the method of Folch. Separation of the lipids will be accomplished by means of thin-layer chromatography on silica gel according to the method of Freeman and West. Radioactivity of the eluate

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will be determined in a scintillation vial and counted in a Tri-carb liquid scintillation spectrometer. The method of Zak will be used for the determination of cholesterol. Phospholipids are analyzed according to the method of Lowry modified by Wagner. The blood vessels will be analyzed for lipid synthesis as well as for cholesterol and cholesterol esters uptake for reasons described (to establish the ratio of ^{14}C cholesterol over ^3H cholesterol). Statistical analyses will be used to evaluate the results.

In the series where the effect of hypoxia will be studied, one vessel from the same patient or the same animal will be perfused with plasma previously made oxygen free, the other artery will be perfused at normal oxygen saturation and tension. The analytical procedures will be identical to those described above. For the hemodynamic experiments, which will deal with an investigation of the effect of changes in the Reynolds number and pressures and flow, alterations in the Reynolds number will result from partial constriction of the perfused vessel, so as to produce turbulence above and below the vessel. The Reynolds number will be determined as described above.

Pressures and flow can be altered by devices inherent in the mechanism of the pump.

Significance of this Work

Studies presented in the progress report have demonstrated that carbon monoxide in various concentration increases cholesterol transport from perfusate into the wall of human coronary arteries. The significance of the planned work is to investigate mechanisms by means of which this

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is brought about. Consequently, although the main thrust of the previous project was directed toward the effect of carbon monoxide, the finding thus obtained now makes possible an investigation of the more general implications of the effect of carbon monoxide on the production of atherosclerosis in human and animal coronary arteries.

For the first time coronary arteries from species other than man can be employed by virtue of the fact that the calculation of the ratio of ^{14}C cholesterol to ^3H -cholesterol in the vascular wall makes possible determinations of the amount of cholesterol transported into the coronary arteries of animals regardless of whether or not cholesterol is synthesized. A separation of chemical and hemodynamic factors which may influence cholesterol transfer appears to be of particular importance. Chemical factors responsible for cholesterol transport can be studied by partial destruction of the vascular wall by means of collagenase; the effects thus obtained on cholesterol transfer can be compared with those of hypoxia. The hemodynamic effects are important because they too, in addition to CO, may play a role in the production of atherosclerosis.

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#39-MASON

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October 18, 1973

Grant application No. 939
CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson, and Wyatt

Subject: R. G. Mason, M.D., Ph.D., Professor, University of
North Carolina
New application No. 939
"Effects of Nicotine on Interactions of Platelets and
Endothelial Cells"

History

This proposal was Case #224 and formal application
was encouraged.

Request

Application No. 939 requests \$41,995, plus two
additional years.

Document Submitted

Attached is application dated August 9, 1973.


F.W.N.

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Encl.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

AUG 29 1973

Date: August 9, 1973

1. Principal Investigator (give title and degrees):

R. G. Mason, M.D., Ph.D., Professor

2. Institution & address:

University of North Carolina
Chapel Hill, N. C. 27514

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Effects of nicotine on interactions of platelets and endothelial cells

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: December 31, 1976

7. Brief description of specific research aims:

We propose to test the hypothesis that nicotine influences the reactivity of endothelial cells with platelets and of platelets with each other. Research in four interrelated areas will be performed:

- a. Comparison of ultrastructure, protein and glycoprotein components, and reactivity with platelets of endothelial cells recovered from human artery, peripheral vein or human umbilical cord and of those harvested from tissue culture.
- b. Development and standardization of an in vitro model for endothelial cell-platelet adhesion. This model will be used in a study of the effects of nicotine, platelet aggregating agents, certain enzymes, and other agents on reactivity of endothelial cells with platelets. The basic approach will be to determine what is required to render endothelial cells attractive to platelets.
- c. Investigation of possible "endothelial supporting" function of platelets and how this may be influenced by nicotine.

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7. (cont.)

- d. Characterization and further studies of an agent of endothelial cell origin which inhibits aggregation of platelets induced by a wide spectrum of agents. Effects of nicotine on the levels, release, and reactivity of this inhibitor will be studied.
- e. In vivo studies of endothelial cell-platelet interaction in the microvasculature of hamster and spider monkey with investigation of effects of nicotine, aggregating agents, anticoagulants, antithrombotic agents, and certain enzyme preparations.

In all of the proposed studies, emphasis will be placed on correlation of function with ultrastructure. The investigations will be carried out by a group composed of workers with special training in the areas of cell biology, pathology, biochemistry, biophysics, and electron microscopy.

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8. Brief statement of working hypothesis:

An understanding of the functions of endothelium in prevention of thrombosis should permit more effective control of this major health problem. Endothelium is thought to present a surface highly compatible with blood. Whether this property of endothelium resides in the molecular constitution of the outer layers of the cell's plasma membrane or is brought about by various inhibitors released by these cells, or both, is unknown. Knowledge of the molecular composition of the endothelial cell plasma membrane gained by disc electrophoretic studies of surface glycopeptides released by treatment with proteolytic enzymes should assist in synthesis of biomaterials more compatible with blood than those currently available. Possible effects of nicotine on membrane components of endothelial cells or platelets could alter their mutual reactivity. Similar benefit should derive from sodium dodecyl sulfate (SDS) gel electrophoretic studies of endothelial cell constituents which will characterize cell proteins and permit identification of those proteins exposed to the outer surfaces of the plasma membrane. Knowledge of conditions under which platelets may adhere to endothelial cells should permit identification of agents to inhibit the phenomenon. Comparison of studies with endothelial cells recovered from umbilical cord vein with endothelial cells grown in tissue culture should be most productive and helpful in the proposed studies since the former do not adhere to platelets while the latter are said to do so.¹ Finally, knowledge of substances of endothelial cell origin which inhibit platelet aggregation or adhesion and effects of nicotine upon these should prove

9. Details of experimental design and procedures (append extra pages as necessary)

A. Experimental design and procedures are listed in each of five interrelated areas of research.

1. Comparison of endothelial cells recovered from human artery, peripheral vein, and umbilical cord vein with endothelial cells grown in tissue culture. Endothelial cells from these 3 sources will be compared ultrastructurally and for differences in electrophoretic mobility, protein and glycoprotein composition, and reactivity with platelets in both the presence and absence of nicotine. These initial studies will be performed to delineate detectable differences between the three types of endothelial cells and detectable effects of nicotine. Only limited studies can be performed with cells recovered from artery or peripheral veins due to obvious difficulties in obtaining specimens within 1 to 2 hours after death. Human umbilical cords can be obtained easily in quantities of 2 to 3 per day, hence endothelial cells recovered from umbilical cord veins will be available for most studies. Endothelial cells of umbilical cord vein and of arterial or peripheral vein origin which have been grown in tissue culture will be available in numbers adequate for all proposed studies.

Representative samples of the 3 types of endothelial cells will be examined in the presence and absence of nicotine by transmission electron microscopy (TEM) for comparison of ultrastructure. Comparison will also be made with the ultrastructure of endothelial cells in situ in various arteries, peripheral veins, and umbilical cord vein. The 3 types of endothelial cells will be compared also by freeze-etch (FE) techniques. Special attention in FE studies will be paid to the outer surface of the plasma membrane of each cell type and a careful search will be made for surface membrane associated particles.

Use of endothelial cells recovered from umbilical cord vein by collagenase treatment will provide useful contrast with use of endothelial cells grown in tissue culture. Platelets do not adhere to endothelial cells recovered

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8. (cont.)

valuable not only in furthering our understanding of mechanisms of thrombosis but also in our search for more effective antithrombotic agents for clinical use.

9. (cont.)

from umbilical veins even if aggregating agents are added, while they are reported to react with tissue culture endothelial cells under such conditions.¹ Effects of nicotine on cellular interactions will be determined. Comparison of protein and glycoprotein SDS gel disc electrophoretic patterns obtained from homogenates of each of the various types of endothelial cells will be carried out and effects of nicotine determined. This will permit detection of loss or gain of components of cells grown in tissue culture as well as detection of differences in patterns of samples from cells isolated by use of collagenase (umbilical cord vein cells) or by use of trypsin (vena cava cells). Further, proteins exposed on the outer surface of endothelial cells now can be labelled and identified.

The aggregometer will be used to compare further the reactivity of the 3 types of endothelial cells with platelets and effects of nicotine on their reactivity. Each endothelial cell type will be stirred at 1000 RPM at 37°C with citrated or heparinized platelet-rich plasma (PRP) or with plasma-free platelets suspended in Ca^{++} - Mg^{++} -free Tyrode's solution (modified Tyrode's solution or MTS). Mixtures will be checked photo-metrically for adhesion of endothelial cells to platelets during stirring and by phase-contrast microscopy at the termination of stirring.

Comparison of the 3 types of endothelial cells in the presence and absence of nicotine will provide information on at least some of the alterations which may occur in these cells as they are isolated from blood vessels and as they grow in tissue culture. This should permit more judicious use of tissue culture endothelial cells in future work.

Extension of studies of tissue culture endothelial cells and effects of nicotine in future years would include work to develop conditions for tissue culture growth of endothelial cells from adult venous and arterial segments from lung, liver, and spleen.

2. In Vitro Interactions of Endothelial Cells and Platelets.

a. Design and standardization of a model for endothelial cell-platelet interaction. The model consists of mixing endothelial cells in MTS with platelets in plasma or with isolated platelets in MTS. The mixture is stirred at 1000 RPM at 37°C in an aggregometer and changes in light transmitted through the mixture are detected by a photocell in the instrument. The following will be investigated:

- 1) Effects of alteration of speed of stirring over the range 100 to 2000 RPM.
- 2) Effects of alteration in temperature. Experiments will be conducted with cell mixtures at 15°, 23°, and 37°.

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- 3) Effects of alteration in pH by use of appropriate buffers over the range pH 5-10 (barbital-sodium acetate buffer, pH 3-9; citrate-phosphate-borate buffer, pH 2-12; phosphate buffer, pH 5-8; imidazole buffer, pH 7.2-9).
- 4) Effects of alteration in ionic strength. Molar NaCl will be added in amounts sufficient to alter ionic strength over the range of normal to 3 times normal. One hundredth molar NaCl will be added in quantities sufficient to lower ionic strength over the range from normal to one-fourth normal.
- 5) Effects of various surfaces. Tests will be conducted in glass, glow discharge-treated glass, polyethylene oxide-treated glass, silicone-coated glass, polycarbonate, and styrene tubes and with Teflon, polyethylene, or poly (vinyl chloride)-covered stirring bars. Platelets are known to adhere to various artificial surfaces and such adhesion may produce release of various platelet components such as ADP and serotonin.

In each of the above, studies will be conducted both in the presence and absence of low levels of nicotine.

These studies should permit standardization of the model (hereafter referred to as the model system) and give us knowledge of which variables we should monitor most closely in future work.

- b. Effects of platelet aggregating agents on endothelial cells and on interaction of platelets and endothelium. Endothelial cells of each type (cells isolated from umbilical cord vein or grown in tissue culture) will be incubated at 37°C without stirring with the following aggregating agents: ADP (10^{-3} to 10^{-7} M), collagen, epinephrine (10^{-3} to 10^{-7} M), serotonin (10^{-3} to 10^{-7} M), or thrombin (0.05 to 5 Iowa units/ml). Following this incubation period, the cells will be added directly to stirring PRP, except in work with higher levels of thrombin, or they will be washed 3 times in MTS and then added to stirring PRP to determine if adhesion of platelets to treated endothelial cells occurs. Likewise, platelets will be incubated with aggregating agents at 37°C without stirring and experiments similar to those described above will be performed. Platelet aggregation and platelet-endothelial cell adhesion will be detected by the aggregometer. Adhesion of platelets to endothelium will be evaluated also by phase-contrast microscopy. If only adhesion of platelets to endothelium and not aggregation of platelets occurs, this can be followed by alteration of light transmission in the aggregometer. If platelets aggregate in addition to adhering to endothelium, adhesion can be monitored by phase-contrast microscopy. In each case, the platelets and endothelial cells will be examined by TEM and by FE if adhesion occurs. The above tests will be conducted both in the presence and absence of nicotine in order to determine whether this agent influences platelet-platelet or platelet-endothelial cell interaction. Further experiments will be conducted with PRP from patients congenitally deficient in fibrinogen, Factor VII, Factor VIII, Factor IX, Factor X, or Factor XII, with PRP from patients receiving dicumarol therapy, and with PRP from patients homozygous for von Willebrand's disease to determine any possible role of the

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intrinsic coagulation system or von Willebrand's factor in the adhesion reaction. In the same manner, PRP from patients with thrombasthenia will be studied to determine reactivity of these defective platelets with endothelium. Similarly, studies will be conducted with platelets isolated by gel filtration and suspended in MTS in an attempt to identify cofactors for the reaction such as divalent cations or fibrinogen. The latter studies will be conducted by use of purified preparations of albumin, fibrinogen, and gamma G globulin. These studies will extend preliminary work reported in Appendix I.

- c. Effects of enzymatic alteration of surface constituents of endothelial cells or platelets. Endothelial cells or isolated platelets in citrated MTS will be incubated at 37°C in the presence and absence of nicotine with low concentrations of the following: neuraminidase (1-100 units/ml), thrombin (0.01-10 Iowa units/ml), trypsin (.01-1 mg/ml), papain (0.1-10 mg/ml), plasmin (0.05-5 mg/ml), or collagenase (0.05-2.0 mg/ml). Following incubation the endothelial cells or platelets will be washed 4x in MTS, resuspended in MTS, and evaluated in the model system for the presence or absence of adhesion between cell types. Neuraminidase removes sialic acid thus reducing the overall net negative charge on cells. Trypsin and papain remove polypeptides and glycopeptides from the outer layers of the plasma membrane. Plasmin attacks fibrinogen, a platelet plasma membrane constituent which plays an important role in aggregation reactions. Collagenase is of interest primarily because of its use in recovery of endothelial cells from the umbilical cord vein. The enzyme preparations to be used will be purified but not entirely free of contaminants, a fact to be remembered in evaluation of data obtained in this work.

Polypeptides and glycopeptides liberated from both endothelial cells and platelets by treatment with enzymes, especially collagenase, thrombin, papain, and trypsin, will be characterized by polyacrylamide gel disc electrophoresis of supernates of enzyme-treated cells. Further, proteins obtained by SDS treatment of control and enzyme-treated cells can be characterized by electrophoresis in SDS containing gel. Proteins exposed on the outer surface of the endothelial cell or platelet plasma membrane can be labelled before exposure to enzymes to detect possible alteration in membrane geometry produced by enzyme treatment. This procedure will be used to determine whether the contractile protein of endothelial cells and platelets is exposed in part on the outer surfaces of these cells.

Such studies hopefully will form the basis for future work on identification of surface entities responsible for nonadhesion of endothelial cells and platelets and for the high degree of blood compatibility of the endothelial cell. Studies similar to those planned here have been reported previously for platelets² and for amoebae.³ At the least we shall seek a correlation between loss of peptides and alteration of endothelial cell-platelet reactivity. At the most, we could gain a clear understanding of protein and saccharide components of the outer reaches of the platelet plasma membrane.

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In addition, when sufficient numbers of endothelial cells are available, we shall isolate cell membrane fractions in the presence and absence of nicotine by a glycerol loading technique and characterize the component proteins before and after enzyme treatment by use of SDS polyacrylamide gel disc electrophoresis. Use of dithiothreitol with SDS will permit more refined critical evaluation of disc electrophoretic patterns and changes which may be produced in these. Staining gels with periodic acid-Schiff (PAS) will permit detection of alteration in glycoprotein components.

Enzyme-treated cells will be studied also by whole cell electrophoresis to detect alteration in surface charge and by TEM and FE to search for ultrastructural alterations. Especially sought for will be alterations of membrane associated particles seen with FE techniques. In later phases of the proposed work we plan to investigate effects of certain other enzymes on interaction of endothelial cells and platelets. Such enzymes include chymotrypsin, elastase, hyaluronidase, acid and alkaline phosphatase, and phospholipase C.

- d. Effects of miscellaneous agents on endothelial cell-platelet interaction. In the model system, a number of diverse agents will be evaluated for their effect on the interaction of endothelial cells and platelets in the presence and absence of nicotine. These agents include: heparin, protamine, polylysine, Reptilase, Arvin, and early and late fibrin degradation products (FDP). Heparin has a strong negative charge in addition to being an effective anticoagulant; and protamine, being positively charged, is a potent heparin-neutralizing agent. Polylysine is a positively charged polymer which reacts with cell surfaces. Reptilase and Arvin alter fibrinogen. All of these agents including FDP affect blood coagulation, or platelets, or both, and therefore may well influence endothelial cell-platelet interaction. Our chief aim is to determine conditions under which the two cell types react.
- e. Inhibition of endothelial cell-platelet adhesion. Under conditions in which endothelial cells and platelets are found to adhere, we plan to investigate the effects of a wide spectrum of agents which belong to the general class of antithrombotic drugs. Each agent will be incubated with endothelial cells or platelets at 37°C for 1 min and for 30 min prior to use of the treated cells in the model system. Agents to be tested include: aspirin, atropine, sulfinpyrazone, phenylbutazone, cyproheptadine, imipramine, reserpine, phentolamine, dibenzylamine, cocaine, parachloromercuribenzoate, promethazine, and prostaglandin E₁. All of these agents, representing various pharmacologic classes, have been reported to inhibit platelet aggregation, and each will be tested in the concentration range 10⁻³ to 10⁻⁸ M. Effects of nicotine on agents inhibiting platelet-endothelial cell adhesion will be determined.

Agents which inhibit adhesion of endothelial cells and platelets will be evaluated further by means of TEM and FE for possible effects on ultrastructure of both cell types. Use of both TEM and FE has been found most helpful in past evaluation of antithrombotic agents.⁴

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- f. Further studies will include investigation of the possible role of leukocytes or erythrocytes in endothelial cell-platelet reactions in the model system. Little is known of possible interactions of leukocytes and platelets, although in the rabbit it has been shown that these cells can play a role in release of vasoactive amines from platelets.⁵ Initial studies will involve addition of buffy coat components to the model system in the presence and absence of nicotine to determine whether leukocytes affect the platelet-endothelial cell reaction. Erythrocytes in small numbers will also be added to the model system for determination of their possible effects.
3. Investigation of Possible "Endothelial Supporting" Function of Platelets. Endothelial cells grown in tissue culture with or without addition of platelets to the culture medium will be evaluated for reactivity with isolated platelets or platelets in plasma in the model system. Studies will be conducted both in the presence and absence of nicotine. Protein patterns on gel disc electrophoresis will be compared using SDS-solubilized preparations of each of the two cell populations. Each cell population will be examined at intervals during growth for ultrastructural evidence of platelet incorporation into endothelial cells.
4. Studies of an Endothelial Cell Component Which Inhibits Platelet Aggregation. Endothelial cells recovered from umbilical cord vein contain a substance which inhibits platelet aggregation. This activity, possibly a polypeptide, is heat labile (90°C, 10 min) and has an apparent molecular weight in the range 1600 to 6000. This antiaggregating activity can be detected easily by use of the aggregometer.
- a. Physical properties of the inhibitor. We propose to investigate further this activity by the following studies:
- 1) Preliminary purification studies by use of filtration through membranes of known porosity as well as use of classical ammonium sulfate fractionation and Sephadex G25 or G50 chromatography. We are limited somewhat by the small quantities of starting material available, but we can obtain even now samples of sufficient volume for preliminary work. Later it is hoped that sufficient quantities of tissue culture endothelial cells can be produced to permit further studies of the inhibitor. This inhibitory activity is released from endothelial cells by freeze-thawing or by treatment with mixtures of platelets and aggregating agents.
 - 2) Gel disc electrophoresis of semipurified fractions to define contaminants and identify electrophoretic properties of the activity. Gel columns can be sliced to isolate component bands; each band or disc can be tested for antiaggregant activity by use of the aggregometer.
 - 3) Further purification can be carried out by use of appropriate Sephadex chromatography and Geon-Pevicon electrophoresis. Further purification steps will depend on the nature of the inhibitory activity, its concentration in cells, and its stability.

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- b. Investigation of mechanisms of release of the inhibitor. Further studies will be carried out to define conditions under which the inhibitory activity is released from endothelial cells. The aggregating agents thrombin, ADP, epinephrine, serotonin, and collagen, in a wide concentration range (vida supra) will be incubated in the presence and absence of nicotine with freshly isolated endothelial cells and with endothelial cells grown in tissue culture. Subsequently, cells will be removed by centrifugation and the supernatant solutions assayed for ability to inhibit aggregation of platelets in plasma induced by standard concentrations of the aggregating agents. Cofactors for release of inhibitory activity such as divalent cations or certain plasma proteins will be sought. Homogenization of endothelial cells will be used to obtain 100% release of inhibitor. Inhibitors released by homogenization will be incubated with aggregating agents prior to their addition to platelets. Temporal studies will be conducted with a range of concentrations of aggregating agents and inhibitors to characterize the type of inhibition which occurs. Efforts will be made to determine effects of the inhibitor on aggregating agents and on platelets. Possible effects of low levels of nicotine on content and release of the inhibitor will be investigated as will effects of nicotine on the actions of the inhibitor. Endothelial cells will be examined by TEM following release of the inhibitor in an effort to determine intracellular storage sites. Platelets will be examined by TEM and FE before and after exposure to the inhibitor and a search will be made for ultrastructural alteration, especially in the plasma membrane.
- c. Other possible effects of the antiaggregant activity. Antiaggregant activity released from endothelial cells will be tested for its effect on adhesion of platelets to glass or collagen, clot retraction, "phagocytosis," adhesion of platelets to endothelial cells, and the platelet release reaction. In addition possible effects of the inhibitor on the intrinsic blood coagulation system will be sought by mixture of the inhibitor with normal plasma. Tests will be conducted both in the presence and absence of nicotine in each case.
5. In Vivo Studies. In vivo studies of the interaction of endothelial cells and platelets and effects of nicotine on these interactions will be conducted in the microvasculature of the hamster cheek pouch. Later, if studies in a nonhuman primate are deemed necessary, similar studies can be conducted in the microcirculation of the mesentery of the spider monkey. The following investigations will be conducted:
- a. Effects of nicotine, ADP, epinephrine, or serotonin applied to venules iontophoretically or of thrombin infused in dilute solution. Concentrations of aggregating agents will be chosen to produce a standard nonocclusive mural thrombotic deposit. Preliminary investigation indicates that this approach is feasible. Careful studies will be performed to determine optimal concentrations and rate of delivery for each aggregating agent. Effects of nicotine on formation of the standard mural thrombus will be studied. This model for mural thrombosis will be characterized thoroughly by TEM to elucidate relationships of endothelial cells, basement membrane and other vascular wall components, and platelets. The model will be based on endothelial cell-platelet adhesion, not connective tissue-platelet adhesion.

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- b. Effects of infused enzymes on the model for mural thrombosis.
Infusion of low concentrations of neuraminidase, trypsin, papain, plasmin, Arvin, or collagenase will be made to determine if removal of sialic acid, surface glycopeptides, or fibrinogen will alter the mural thrombus model. Collagenase and trypsin will be tested primarily to determine effects they may have on reactivity of endothelial cell-platelet interaction and will be correlated with similar in vitro studies. If altered endothelial cell reactivity results from enzyme treatment, studies will be conducted to determine effects of nicotine on this altered reactivity.
- c. Use of the mural thrombus model to test for effects of anticoagulant and antithrombotic agents. The antiaggregating activity of human endothelial cell origin will be assayed in the mural thrombus model in both hamster and spider monkey. If the inhibitory activity is effective under conditions of the test, the mural thrombus model could serve as a bioassay, and effects of nicotine on actions of the inhibitor in vivo would be sought. Additionally, heparin and the antithrombotic agents listed in "2.e." above will be tested for their effects on endothelial cell-platelet adhesion in the in vivo mural thrombus model. Anti-aggregants will be given systemically, and a wide concentration range of each agent will be investigated. Effects of test agents will be monitored by light microscopy, cinephoto-micrography, and, in selected cases, by TEM.

B. Methods of Procedure.

We have entered into several aspects of research on endothelium during the past year. Dr. David Sharp, an intern and graduate student in experimental pathology in our department, spent the 1971-72 academic year working in the laboratory of Dr. G. V. R. Born in London. Under Dr. Born's supervision, Dr. Sharp has mastered the technique of iontophoresis⁶ and learned to apply this in studies of the microcirculation of the hamster cheek pouch. Dr. Sharp has studied the ultrastructure of endothelium exposed to iontophoretically applied aggregating agents such as adenosine diphosphate (ADP).^{7,8} In addition, he spent one month in the laboratory of Dr. Arfors in Uppsala learning additional techniques used in research on the microcirculation.⁹

Dr. Sabiha Saba, long a member of our research group, began in July, 1972 a study of endothelial cells recovered from several sources. She has successfully recovered endothelial cells from segments of canine aorta by use of low concentrations of trypsin. She has also recovered endothelial cells from human umbilical cord vein by use of collagenase and has used these cells in aggregometric studies with platelets in plasma and with isolated platelets. These studies, presented in an accompanying manuscript, show that platelets in plasma do not adhere to endothelial cells recovered from umbilical cord vein even if adenosine diphosphate (ADP) or epinephrine is added. Such endothelial cells do not adhere to platelets isolated from plasma even in the presence of added thrombin, ADP, or epinephrine. Further, human endothelial cells exposed to platelets and aggregating agents release a small molecule of apparent 1400 to 16000 molecular weight, possibly a polypeptide, which is an effective inhibitor of platelet aggregation. This inhibitor is inactivated at 90°C for 10 min.

We have established successful tissue culture of endothelium of human umbilical cord vein origin in our laboratory. This was accomplished by Dr. Saba and Dr. Mohammad. Dr. Ralph Nachman generously permitted Dr. Mohammad

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to visit his laboratory and receive limited but highly useful instruction in tissue culture of endothelium. In addition, Drs. Saba and Mohammad received informal training in tissue culture techniques in the laboratory of Dr. D. O. Adams, Department of Pathology, Duke University, Durham, N. C.

Methods and materials are listed alphabetically:

1. Adhesion of platelets to collagen will be quantitated by addition of standard amounts of human collagen of Achilles tendon origin prepared by the method of Hovig¹⁰ to PRP in the aggregometer. An increase in light transmission is produced by adhesion of platelets to collagen when ethylenediamine tetraacetic acid (1%) is used as anticoagulant. Results will be monitored by phase-contrast microscopy also.
2. Adhesion of platelets to glass will be quantitated by the method of George et al.¹¹
3. Adenine nucleotide content of platelets will be measured by the luciferase reaction using a Biometer.¹²
4. Aggregating agents. Human thrombin prepared by the method of Miller and Copeland¹³ will be further purified by the method of Kerwin and Milstone.¹⁴ Human Achilles tendon will be used to prepare "collagen" by the method of Hovig. ADP, epinephrine, and serotonin will be purchased from Sigma Chemical Co. All aggregating agents will be dissolved in 0.08 M phosphate buffer, pH 7.35, in 0.145 M NaCl since Tris buffer produces undesirable ultrastructural changes.
5. Aggregometer. Three Chrono-Log aggregometers are in use in our laboratories.¹⁵ Aggregometer tracings will be analyzed by the techniques of Frojmovic.¹⁶ In addition, samples can be monitored by use of phase-contrast microscopy and, when warranted, by TEM.
6. Blood. Donors of normal blood will be carefully selected students and young employees of both sexes. Donors are rejected if they receive any type of medication regularly or if they have taken aspirin within the preceding week. Donors congenitally deficient in fibrinogen or blood coagulation factors VII, VIII, IX, X, or XII as well as patients with von Willebrand's disease or thrombasthenia are readily available through our Clinical Coagulation Center. Dogs congenitally deficient in factors VII, VIII, or IX also are readily available at the department's Frances Owen Blood Research Lab. Patients receiving dicumarol therapy are available through our Clinical Coagulation Laboratory.
7. Chromatography with Sephadex G25 or G50 will be conducted as described by Fisher.¹⁷ Gel filtration chromatography of platelets will be carried out by the method of Tangen et al. using MTS as elution fluid.¹⁸⁻²⁰
8. Clot retraction of PRP will be conducted by a previously described method.⁴
9. Electron microscopy. TEM of cells fixed in situ or in suspension will be carried out by previously described methods²¹ using buffered 4/3 or 8/3% glutaraldehyde as fixative. FE methods have been described and are those of Reddick and Mason²² and of Mühlethaler.²³ The Balzers freeze-etch apparatus²⁴ in the nearby Department of Botany is available to us on a rental basis.

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10. Electrophoresis and Study of Proteins and Glycoproteins. Electrophoretic mobility of intact cells will be performed by the method of Seaman.²⁵ Disc electrophoresis in 5% or 7% polyacrylamide gels with barbital buffer and with or without addition of sodium dodecyl sulfate will be carried out as described by Gordon²⁶ using Comassie blue stain for protein and periodic acid-Schiff stain for glycoprotein. Molecular weight estimations will be carried out as described by Weber and Osborn²⁷ and by Mann et al.²⁸ Electrophoresis in Geon-Pevicon will be by the method of Muller-Eberhard.²⁹ Lactoperoxidase iodination of exposed membrane protein will be carried out by the method of Phillips and Morrison.³⁰
11. Endothelial cells. Human endothelial cells will be recovered from umbilical cord vein by use of low concentrations of collagenase and from vena cava by use of dilute trypsin. Umbilical cords are being obtained daily from the Obstetrical Services of N. C. Memorial Hospital, Chapel Hill, N. C., Watts Hospital, Durham, N. C., and Alamance County Hospital, Burlington, N. C. Tissue culture of endothelial cells from cord or vena cava will be conducted by methods described by Nachman;³¹ these have proven successful in our hands. Human vena cava segments can be obtained within one hour after death from cadavers authorized for organ donations.
12. Enzymes. Arvin (Twyford Labs, London) and plasmin (Schwarz-Mann) will be purchased from commercial sources. Collagenase, neuraminidase, papain, trypsin, and other listed enzymes will be purchased from Worthington Biochemicals.
13. Glassware will be coated with polyethylene oxide as described by Hiatt et al.³² or with silicone as previously described.³³ Glow discharge treatment of glass will be carried out gratis at Research Triangle Institute, Research Triangle Park, N. C.
14. Iontophoresis will be carried out by methods described by Duling et al.⁶
15. Membrane preparations from endothelial cells will be obtained by the method of Barber and Jamieson.³⁴
16. Miscellaneous. Antithrombotic agents are all on hand; additional supplies can be purchased from Sigma Chemical Co. or obtained gratis from appropriate pharmaceutical firms. Fibrin degradation products will be prepared by the method of Marder et al.³⁵ Heparin will be "Liquaemin" from Organon Labs. Reptilase (Pentapharm, Inc.) is on hand.
17. Microvasculature. Procedures used for study of the microvasculature of the hamster cheek pouch³⁶ or spider monkey mesentery³⁷ have been described. Animals will be housed in the medical school's Division of Laboratory Animal Medicine and all work with animals will be conducted in accordance with NIH regulations.
18. Nicotine as nicotine HCl will be prepared in 0.05 M phosphate buffer, pH 7.35 in 0.154 M NaCl.
19. Phagocytosis of latex particles by platelets will be conducted as described by Movat et al.³⁸

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20. Platelets will be prepared as suspensions in citrated plasma at 23°C as previously described.³³ Platelets separated from plasma by gel filtration will be prepared by the method of Tangen et al.¹⁸ We have recently found these platelets superior to those prepared by a number of other methods.³⁹
21. Release reaction of platelets will be followed by measurement of adenine nucleotides using the luciferase reaction,¹² or by use of C¹⁴ labelled serotonin.⁴⁰
22. Tissue culture of endothelial cells will be conducted by methods described by Nachman.³¹

All of the above methods are being used or have been used by us except the iodination of cell membrane proteins.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Our research group, currently occupying 2000 sq. ft. of research space will move in December, 1973 into 2800 sq. ft. of new research space in the Preclinical Education Building. This space will be equipped with fume hoods, walk-in temperature-controlled rooms, and an electron microscopy suite for our RCA-EMU3G transmission electron microscope and Quicksan scanning electron microscope. Our group now has on hand a variety of centrifuges including a Spinco preparative ultracentrifuge, various pH meters and balances, a Nuclear Chicago 3 channel scintillation counter, gel electrophoresis apparatus of several types, equipment for column or thin layer chromatography, equipment for immunodiffusion, and immunoelectrophoresis, the Seaman apparatus for electrophoresis of intact cells, several phase-contrast microscopes, three aggregometers and recorders, gradient maker and ultraviolet monitors for chromatography, laminar flow hood and atmosphere-controlled incubator for tissue culture, an ultrasonic probe, drying ovens, various pumps and temperature-controlled baths, refrigerators, -20° and -40 to -70° freezers, vacuum evaporators, complete dark room facilities, micromanipulator and some necessary optics for microvascular studies, and a Zeiss PMQII spectrophotometer. Available within the department are complete facilities for amino acid analysis (Beckman) and for column electrophoresis and electrofocusing (LKB).

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

% time

Amount (including social security, retirement, and insurance)

Mason, R. G., M.D., Ph.D., Principal Investigator

10

Saba, S. R., M.D., Coinvestigator

30

3846

Chuang, H. Y. K., Ph.D., Coinvestigator

20

3712

Mohammad, S. F., Ph.D., Coinvestigator

30

3502

Sharp, D. E., M.D., Coinvestigator

20

Reddick, R. L., M.D., Coinvestigator

20

Technical

Perry, J. M. (technician)

100

9652

Bazan, M. E. (lab helper)

50

3050

Smith, M. (secretary)

25

1770

Sub-Total for A

25,532

B. Consumable supplies (by major categories)

General chemicals

700

Biochemicals including enzymes

1600

Human blood (50 units at \$15/unit)

750

Tissue culture supplies

1800

Electron microscopy and photographic supplies

800

Animal purchase and board

480

Sub-Total for B

6130

C. Other expenses (itemize)

Mileage (\$0.10/mile; 2000 miles/yr for procurement of umbilical cords from cooperating hospitals)

200

Copying

100

Communications

100

Glass shop and electronics shop charges

200

Service contracts (scintillation counter, 25%, \$225; electron microscope, 25%, \$900)

1125

Sub-Total for C

1725

Running Total of A + B + C

33,387

D. Permanent equipment (itemize)

Payton aggregometer

1900

Beckman recorder

1100

Miscellaneous (trip balance, table top centrifuge, water bath)

600

Sub-Total for D

3600

E. Indirect costs (15% of A+B+C) 15% of \$33,387

E

5008

Total request

41,995

15. Estimated future requirements:

| | +10% yearly Salaries | +5% yearly Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------------------|------------------------------|----------------|------------------|----------------|--------|
| Year 2 | 28,085 | 6,437 | 1725 | 1000 | 5437 | 42,684 |
| Year 3 | 30,894 | 6,759 | 1725 | 1000 | 5907 | 46,285 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|---|-------------|------------------------|
| 1. Role of plasmatic components in blood-solid surface reactions | NHLI, NIH 5K4HE46351 | \$25,000/yr | July, 1970 to July 197 |
| 2. Blood platelets, membranes, aggregation and adhesion | NHLI, NIH, HL13296 | \$21,031 | June 1971 to June 1976 |
| 3. Mechanisms of platelet adhesion and aggregation in thrombosis | NHLI, NIH, HL14228 | \$73,433 | June 1971 to Dec. 1976 |
| 4. Antithrombogenic surfaces: platelet-interface reactions | AKCUP, NIAMDD, NIH PH4368977 (contract) | \$87,358 | Dec. 1972 to Dec. 1975 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--------------------------------|-----------------------|---------------------------------------|
| 1. Pathologic alterations in endothelium and thrombosis | NHLI, NIH | 51,866 (requested) | Jan. 1974 to Jan. 1975 (requested) |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University of North Carolina

Mailing address for checks

W. W. Fulk

Contract Administration

South Bldg, Chapel Hill

Principal investigator

Typed Name R. G. Mason

Signature

Telephone

Area Code 919 Number 966 Extension 1348

Responsible officer of institution

A. H. Shepard, Jr. 23 Aug 1973

M. A. H. Shepard, Jr., Asst. Vice

Typed Name President and Treasurer

G. R. Holcomb, Ph.D., Dean, Res.

Title Admin.

Signature

Shepard: 919-933-1691

Telephone

Holcomb: 919-933-1383

Area Code

Number

Extension

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#952 - McDONALD

1003540249

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 10, 1974

Grant application No. 952

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson and Sommers

Subject: Ted P. McDonald, Ph.D., University of Tennessee
New application No. 952
"Tobacco Smoke and Platelet Function"

History

This proposal was Case 246 and formal application was encouraged.

Request

Application No. 952 requests \$43,911, plus two additional years.

Document Submitted

Attached is application dated 12/14/73 (38 pages).

Comment

We shall probably request evaluation of this proposal by an outside consultant.

F.W.N.

F.W.N.

FWN:wg
Encl.

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Signature copy *ade 1/7/74*
gh

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 121-8885

Application for Research Grant
(Use extra pages as needed)

1973
Date: 12/14/73

Case No. 246

1. Principal Investigator (give title and degrees):

Ted P. McDonald, Ph.D., Research Associate Professor

2. Institution & address:

University of Tennessee Memorial Research Center
1924 Alcoa Highway
Knoxville, Tennessee 37920

3. Department(s) where research will be done or collaboration provided:

University of Tennessee Memorial Research Center
1924 Alcoa Highway
Knoxville, Tennessee 37920

4. Short title of study:

Tobacco Smoke and Platelet Function

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Acute myocardial infarction is a major cause of death and individuals who smoke have an increased risk of cardiac and vascular disorders as compared with nonsmokers. Most of the studies reported in the literature are plagued by small samples, inappropriate controls, use of hospitalized patients and often present conflicting results. The intent of this proposal is to carry out a controlled laboratory study in rats and human volunteers on the effects of tobacco smoke and nicotine on: 1) platelet function, including adhesion, aggregation, clot retraction, and plasma factors; 2) platelet production, including peripheral platelet counts, platelet life span, platelet size, and changes in the bone marrow megakaryocyte population; 3) chemical alterations of platelet mucopolysaccharides, hexosamines and protein content; and 4) determination of thrombopoietin levels in serum from treated animals and human volunteers with the goal of adding to our present knowledge of the involvement of platelets in coronary heart disease and clarifying some of the conflicting and poorly substantiated data present in the literature.

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Coronary insufficiency may be preceded by impairment of blood flow through the vessels near or in the heart itself. This impairment may be due to actual alterations in the endothelial lining of the blood vessels (such as increased atherosclerosis among smokers)¹ or to the sticking of platelets either to one another or to vessel walls, which could partially or completely occlude blood vessels. Increased atherosclerotic vessels are jagged and lose their silicone-like properties,² thus increasing the possibility of rupture and concomitant aggregation of platelets into clumps or thrombi. If platelet clumps are not broken apart they can lodge in blood vessels, stopping the flow of blood. Therefore, one important aspect of determining how smoking causes an increased risk of heart attacks is to examine the effects of smoking on the formed elements of the blood which are intimately involved in vascular blockages - the platelets. Several methods of inquiry will be used to examine the effects that smoking and nicotine have on blood platelets.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Literature review, significance, and reasons for study.

More people die as a result of heart attacks every year than any other disease.³ Smokers have a significantly higher incidence of myocardial infarctions^{1,5} related vascular disorders such as Burger's disorders,⁴ coronary artery disease,^{1,6,7} carcinoma of the lung⁸ and other types of malignancy,⁹ as well as chronic bronchitis and pulmonary emphysema⁶ as compared to nonsmokers. One study indicated that nonsmokers who are in close association with smokers often inhale almost as much cigarette smoke as a smoker.¹⁰ The children of parents who smoke suffer from the toxic effects of cigarette smoke all of their lives whether or not they become smokers themselves.¹⁰ Mothers who smoke during pregnancy block oxygen transport - in direct relation to the smoking habit - across the placental membrane to the fetus which results in an increased number of still-born babies or children with retarded growth and/or development.^{11,12}

(1) Tobacco smoke and platelet activity.

Cigarette smoke has been shown to cause an accelerated thrombus formation *in vitro*,¹³ supporting the finding that smokers have an increased thrombotic tendency. Sixty human subjects both male and female were used with ages ranging from 17 to 68 years. All subjects were patients in a private hospital and all were habitual smokers. Each patient served as his own control and used his own cigarette brand. Blood samples were taken before smoking and 20 min after smoking 2 cigarettes; 34 out of 60 habitual smokers showed accelerated thrombus formation.

Engelberg and Futterman¹⁴ later repeated this work using a similar experimental design, but with a larger sample size (147 individuals). Again, all subjects were habitual smokers and were either hospital patients or staff at the hospital. The reasons for hospitalization of numerous test subjects were not indicated and each person again served as his own control. The results of this study showed 132 of the 147 individuals had an accelerated thrombus formation after smoking 2 cigarettes.

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Mustard and Murphy¹⁵ reported that smoking decreased platelet survival and increased platelet turnover, thereby shortening the platelet life span. In this work only 7 human subjects were used and each had a history of some type of vascular or heart malady: myocardial infarction, cerebrovascular accidents or disorders and all were heavy smokers, i.e. greater than 1 pack per day. Since O'Brien *et al.*¹⁶ found that nonsmoking patients suffering from myocardial infarctions had more active platelets (larger size and greater adhesion) than normal nonsmoking subjects, the above studies are in question.

Platelet adhesiveness has been reported to be enhanced after smoking in studies by Ambrus and Mink,¹⁷ Ashby *et al.*¹⁸ and by El-Ebrashy *et al.*¹⁹; whereas, Mustard and Murphy⁵ and Murchison and Fyfe²⁰ found no such increase after smoking.

A decreased platelet count and decreased blood coagulation time in patients after smoking a single cigarette was reported by Shimamoto.² This decrease was postulated to be due to damage of the endothelial silicone-like property of the blood vessel. Recently, Hawkins²¹ also demonstrated that the blood of smokers had a decreased coagulation time as compared to nonsmokers, as well as increased rates of initial clot formation and clot retraction. Platelets from individuals who smoked were more active than those of nonsmokers when aggregated with ADP. The clot formation and clot retraction data are based on thromboelastography which can measure tensile strength of clots and the rate of clot formation. A different technique has been used recently to demonstrate increased clot retraction of rat blood platelets after nicotine treatment.^{22,29} This technique allowed a determination of weight changes of the retracted clot and seems to be a better method for determining increased function of platelets.

Levine²³ recently presented evidence that smoking a single cigarette caused an increase in the platelet's response to a standard aggregating stimulus in human volunteers. The response appears to be related specifically to the inhaling of tobacco smoke, since it was not observed following the smoking of lettuce leaf filled cigarettes.

(2) Nicotine and platelet activity.

Nicotine is frequently considered to be the causative agent of tobacco smoke in the development of coronary and vascular disease, although this association has not been conclusively proven. Many investigators have examined the effects of nicotine on numerous tissues of the body, but there are relatively few studies on the effects of nicotine on blood platelets.

Wenzel and Singh²⁴ have shown that intravenous injections of nicotine depressed coagulation time in rabbits. Epinephrine likewise decreased coagulation time and an injection of nicotine and epinephrine given together acted synergistically to decrease coagulation time. Earlier work by Wenzel *et al.*²⁵ demonstrated that both oral and intravenous administration of nicotine to rabbits resulted in decreased coagulation time. These findings seem to indicate that the route of administration of nicotine into the body does not affect the action of nicotine on coagulation time.

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Recently, McDonald and Clift²⁶ described increased platelet aggregation in rats after oral nicotine administration when compared to aggregation of platelets from control rats. The increased aggregation appeared to be due to both an alteration of some plasma factor and to changes in the platelets themselves. More experiments are needed using larger samples and standardized platelet numbers to determine the mechanism of alteration.

Werle and Schievelbein²⁷ suggested that platelet aggregation was proportional to the concentration of nicotine used. Unfortunately, in order to show platelet alterations, high concentrations of nicotine had to be administered. They also found that nicotine and ADP enhanced platelet aggregation when added simultaneously. The aggregometer used in these studies required the use of a manual stirrer which could rupture platelets or produce uneven distributions resulting in false aggregation data. By use of a Chrono-log aggregometer with continuous automatic mixing (described in the following methods section), one could more accurately measure and determine the aggregation of platelets exposed to various substances.

One of the most widely used tests of platelet alteration after smoking is platelet adhesion.²⁸ The techniques which are employed have a high degree of variability so the results are questionable. No conclusive data are present in the literature concerning the effect of nicotine on platelet adhesion. Therefore, further investigations, using refined techniques, need to be performed in order to clarify the effects of nicotine on platelet adhesion.

Another method of measuring platelet function is by use of clot retraction. An increase in clot retraction as determined by the weight of the retracted clot has been shown to be a measure of platelet function. McDonald *et al.*²⁹ have recently demonstrated that rats exposed to nicotine (both oral and intravenous) have increased clot retraction (which results in a smaller clot) and were therefore more functional as compared to nontreated controls. One can conclude, therefore, that nicotine ingestion definitely alters certain platelet functions.

(3) Smoking vs nicotine effects

Isaac and Rand³⁰ reported that the amount of nicotine extracted from a single cigarette was concentrated in the plasma of a smoker by a factor of 1.5 to 2.5 depending on the nicotine content of the cigarette. Although the amount of nicotine in the plasma was reduced by one-half 30 min following cessation of smoking, a smoker could accumulate higher and higher concentrations of nicotine by continued smoking.

Smoking and nicotine injections have both been shown to decrease clotting time in dogs. However, nicotine alone did not alter platelet counts or fibrinogen levels but smoking did.³¹ Since tobacco components are altered while the cigarette burns,³² it is very probably that oral or intravenous administrations of nicotine would not simulate tobacco components found in the burning cigarette, thus the necessity of studying the effect of smoke rather than nicotine.

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These findings strongly suggest that while nicotine may account for certain physiological changes associated with smoking, cigarette components other than nicotine may be responsible for various alterations in platelet activity that could lead to heart and vascular malfunctions. Therefore, a comparative study of the effects of nicotine and tobacco smoke on blood platelets needs to be performed.

(4) Need for laboratory study.

Most of the studies to date concerning platelets and smoking have been limited to platelet functions (adhesion, aggregation and clot retraction) and have not dealt with other parameters of platelet activity such as their production and chemistry. In general, results of smoking studies in the literature have considerable experimental error. The majority of studies concerning the effects of smoking on blood platelets have been performed on human subjects, who for the most part, have been categorized depending on their smoking habits as a nonsmoker, a light smoker (1 pack or less/day) or a heavy smoker (more than 1 pack per day). Test subjects are required to smoke cigarettes either *ad libitum* or a predetermined number for a designated period of time. After smoking, blood is taken and various platelet activities are analyzed. Most of the studies use only smokers and therefore have no suitable control group. In addition, to compound the error, subjects are often hospital patients suffering from one or more types of heart and/or vascular disorders in which altered platelet functions have been found.

Futhermore, the use of human subjects also prohibits the investigator from controlling the duration of the cigarette puff, thus varying the doses among the smokers. A standard puff of cigarette smoke, as defined by the British and American tobacco council, consists of a two second puffs of 35 ml of smoke pulled through a cigarette once each minute down to a 30 mm butt length.^{30,33} It seems possible that some smokers may obtain roughly 30-50 times this amount of smoke and other smokers may obtain less thus varying the amounts of smoke constituents such as the amount of nicotine per cigarette.³⁰ If alterations in platelet function, production and chemistry are found as compared to controls, then for the first time one can accurately attribute these changes to treatment rather than to biological variations in health, diets, emotions, different doses, as well as the noxious effects of the various types of pollution than human subjects encounter.

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B. Rationale and Methods

It is planned to study rats after exposure to tobacco smoke or nicotine treatment for altered platelet function (platelet adhesion, aggregation, clot retraction, and plasma factors); for changes in platelet production (peripheral platelet counts, platelet life span, size and megakaryocyte numbers); for chemical alterations of platelets (mucopolysaccharide content, hexosamine values, and amounts of protein); and for thrombopoietin content of sera. In addition, a study of platelets in human volunteers is planned.

(1) Methods of Exposing Animals

(a) Smoking Machine

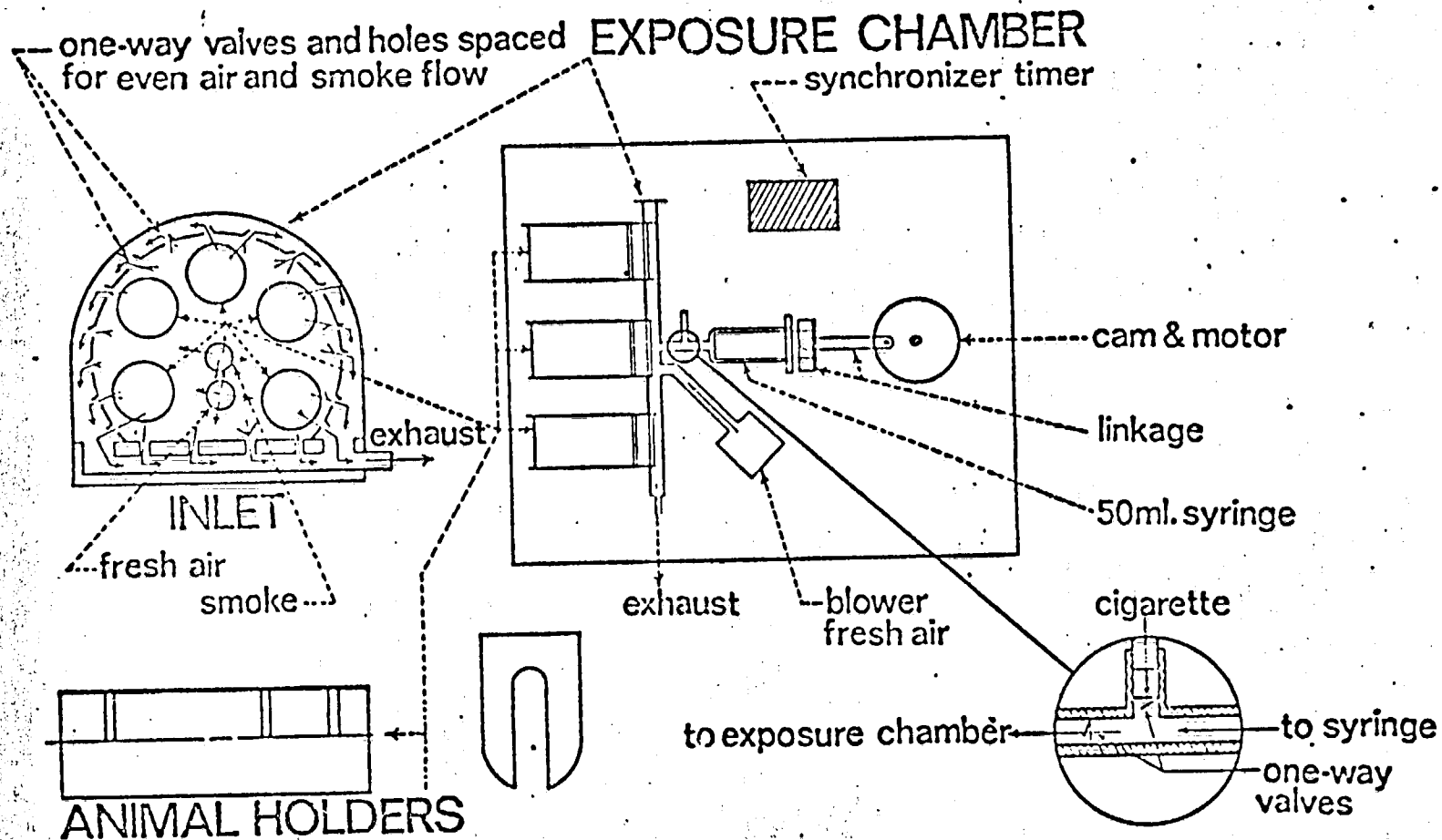
Smoke will be administered to male Sprague-Dawley rats (200-350 gm) by use of a small "Walton" smoking machine purchased from Process Instrument, Inc., 1943 Broadway, Brooklyn, NY. A machine (see attached figure) which is a modification of several other machines will be used until the new machine is available.^{34,35} The Engineering Mechanics Department at the University of Tennessee designed and built this machine according to our specifications.³⁶ The machine delivers a standard puff^{30,33} of cigarette smoke each minute to rats with a minimum amount of smoke dilution. This machine will simultaneously expose 5 rats to standard puffs of cigarette smoke with roughly a 1:7 dilution of smoke with air. Rats are exposed to fresh smoke for 20 seconds after which a blower exhausts the breathing chamber and continues to give the rats fresh air for 30 seconds. The blower then shuts off and coasts to a stop before the next puff cycle begins.

Two types of control groups will be used to insure that the stress of the machine and/or restraint devices do not induce any platelet alterations. One group will remain in their normal cages undisturbed for the 10 day exposure period. Another group of controls will be sham-smoked for as long as the longest treatment groups.

At present, our treatment groups include exposure to either 2, 8, or 16 cigarettes/day for 10 days. These represent light doses in comparison to those obtained by a heavy smoker (> 1 pack/day). These doses may need to be modified in accordance with the increased efficiency of the "Walton" machine.

Since the normal platelet life span in rats is about 4.5 days,³⁹ the rats will be exposed for 10 days to insure that all the platelets examined have been produced under smoking conditions. A standard reference cigarette (1R1 purchased from the University of Kentucky - Tobacco and Health Research Institute) will be used for all experiments to insure a consistent tobacco composition, thus greatly reducing the variability between groups of cigarettes.

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(b) Nicotine Administration

Nicotine (nicotine alkaloid, Sigma Chemical Co.) will be injected intravenously (10-160 µg/kg body wt) or given to rats in their drinking water for 2 weeks prior to assay at concentrations of 25 mg/L, 12.5 mg/L and 0 mg/L. Every day fresh nicotine-water will be prepared and 400 ml placed on rat cages (2 rats/cage). The amount of liquid remaining in the bottles each day will be used to calculate the dose on a mg nicotine/kg body wt/day basis.

(2) Platelet Function Studies

(a) Platelet Adhesion

The adherence of platelets to surfaces such as the endothelial lining of blood vessels is referred to as adhesion. Although platelets normally adhere to subendothelial layers of vessel walls at sites of injury to prevent blood loss, adherence to vessel walls, other than for reasons of maintaining hemostasis, can block the flow of blood. Increased platelet adhesion has been directly correlated with acute venous and arterial thrombosis.^{40,41} Since individuals who smoke have an increased occurrence of various types of vascular occlusions, one effect of smoking might be altered platelet adhesiveness.

In this work a modification of Salzman's method⁴² will be used to determine platelet adhesion. Rats will be anesthetized with ether and 7-8 ml of blood drawn from the dorsal aorta into a 12 ml plastic syringe containing 10 U of heparin per ml of blood. The excess blood over 5 ml will be expressed into a separate tube for routine analysis (platelet count, white blood cell (WBC) count, hematocrit and red blood cell (RBC) count) and utilized for the initial platelet count. The remaining 5 ml will be expressed through a 17.2 cm polyvinyl tubing filled with glass beads (3M Superbrite beads 100-5005) at the rate of 1 ml/15 seconds into a 15 ml EDTA tube for the final platelet count. The percentage of platelets remaining in the glass-bead-filled tube, referred to as the percent platelet adhesiveness, will be calculated by subtracting the final platelet count from the initial count divided by the initial platelet count times 100:

$$\text{Percent Platelet Adhesiveness} = \frac{\text{Initial count} - \text{Final count}}{\text{Initial count}} \times 100$$

By use of this procedure the platelet adhesiveness index for rats is usually between 65-85%. In order to adequately measure an increase in adhesiveness due to smoking one needs to reduce the normal values to around 50%. The adhesiveness index can be reduced by shortening the polyvinyl tube length and/or increasing the flow rate of the blood over the glass beads. If smoking causes significant increases in adhesiveness over nontreated controls, then one should be able to measure these changes using such a modified technique.

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(b) Platelet Aggregation

The adherence of platelets to one another (aggregation) is another important mechanism in maintaining normal hemostatic conditions within the body. Under certain conditions platelets can aggregate and form a thrombus or a clot in blood vessels, which can occlude the vessel and many times lead to a fatal condition. Several substances are known to cause platelets to aggregate: adenosine diphosphate (ADP), epinephrine, collagen, and others. Platelets normally contain ADP, so when a platelet ruptures it releases ADP causing other platelets to rupture which cascades into a platelet aggregate.⁴¹ Nicotine is known to cause increased levels of epinephrine in the plasma which might account for the increased platelet aggregation among smokers.⁵ Levine^{23,43} recently reported that aggregation induced by ADP, epinephrine, or collagen was more pronounced in platelets of cigarette smokers than in nonsmokers.

A Chrono-log Platelet Aggregometer attached to a Chrono-log model 702 recorder will be used to determine platelet aggregation;⁴⁴ ADP, collagen, and epinephrine will serve as the aggregating agents. Platelet rich plasma (PRP) and platelet poor plasma (PPP) will be prepared in the following manner: 8 ml of blood will be drawn from the abdominal aorta of each rat into 12 ml plastic syringes containing 80 U of heparin. The blood will undergo differential centrifugation (107 x g) for 30 min. at 22°C to obtain the PRP and to separate the platelets from the WBC and RBC. The PPP will be prepared by a more rapid centrifugation (760 x g) of the remaining blood for 20 min. Platelet suspension counts using an Electrozone Celloscope will be made so that the concentration of aggregating agent/platelet can be kept constant throughout experiments. A 0.5 ml sample of the PRP and the PPP will be placed into 0.312 inch diameter siliconized cuvettes along with a small teflon stir-bar. While in the aggregometer the platelet preparations will be stirred at a constant rate (1200 rpm) and maintained at 37°C.

Since the operating principle of the aggregometer is based on the interception of light on a photocell (as in a spectrophotometer), the PRP will cause a decrease in transmittance; whereas, the PPP will allow an increase. After determining 10% and 90% transmittance with PRP and PPP, an aggregating agent will be pipetted into the cuvette in order to start the aggregation. A tracing of approximately 5 min will be recorded and the percent aggregation at one, two and three min after the introduction of the agent will be measured by the change in light transmission. The rate of aggregation, the relative size of the aggregates, and the percent aggregation can be determined from the curve which will be plotted. This will enable one to accurately determine some of the effects that smoking might have on platelet aggregation.

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(c) Clot Retraction

ADP release and subsequent release of platelet factor-3 from platelets cause a lengthy series of coagulation reactions which ultimately end in the formation of a clot sealing off the ruptured vessel. The clot then retracts through the action of the contractile-like protein, thrombosthenin, forming the final hemostatic plug. Normally, this clot will be dissolved and removed from the circulation.²² The clots which form in vessels that are not in response to normal hemostatic maintenance and are not dissolved can result in vascular blockage. It is the formation of this latter type of clot which is primarily responsible for myocardial infarctions among smokers. In a recent study, nicotine has been shown to increase clot retraction in rats.²⁹ However, no studies apparently have been performed concerning the effect of tobacco smoke on clot retraction.

Clot retraction studies will be done according to the method outlined by McDonald *et al.*²⁹ PRP will be diluted with PPP to give platelet concentrations of 400,000; 200,000; 100,000; 50,000; 25,000; 12,500; and 6,250 platelets/mm³. To 0.2 ml of each of the above PRP (in plastic Kahn tubes) 0.2 ml of PPP containing 0.1 volume of 0.33 M CaCl₂ will be added. In order to initiate clot formation, 0.05 ml of thrombin (50 U/ml) will be added to each tube and the tubes incubated for 2 hrs in a 37°C water bath. After 10 min the clots will be loosened from the walls of the tube by agitation and returned to the bath. After incubation the clots will be weighed to determine the extent of retraction. If smaller than normal clots are found, then the clot has retracted to a greater extent and is, therefore, more functional. Reduced weight of clots (as compared to normal controls) indicates that the platelets are more active than normal and hence more likely to be involved in vascular occlusions.

(d) Plasma Factors

Previous work by the applicant²⁹ has shown that the effects of nicotine on clot retraction of rat blood platelets were not directly on platelets. It was concluded that the enhanced clot retraction observed in the blood of nicotine-treated animals was due to a plasma factor and not to changes in the platelets themselves.²⁹

As a corollary to the work on platelets, a secondary effort will be focused on the isolation of the plasma factor. Fibrinogen may be a necessary co-factor for ADP-induced platelet aggregation and can be isolated by the use of newer techniques from plasma of animals and humans after smoking.⁵⁹ The extracted fibrinogen can then be used in the determination of specific effects on platelets. In addition, other plasma proteins will be partially purified by electrophoresis and other methods for testing in vitro.

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(1) Detection and quantification of fibrinogen in blood plasma

The method for the detection and quantification of fibrinogen complexes, fibrinogen itself and fibrinogen derivatives in plasma is as follows: columns used are 1 x 30 cm and gel employed is Bio-Gel 5M (Bio-Rad) with an average gel height of 29 cm. Tris saline buffers, [14.5 grams tris (hydroxymethyl)aminoethane, 35 g sodium chloride, pH adjusted to 7.6 with 1 N HCl and made up to 2 liters] will be used both for column equilibration and for gel filtration. All procedures are run at room temperature to avoid difficulties with cryoprotein precipitation. Columns are packed in the conventional manner and then calibrated with known normal plasma with the column flow rate at 12 ml an hour. "Normal" plasma is eluted, as described below, and column gel content adjusted so that under standard flow or elution conditions, fibrinogen patterns are symmetrical at 10 ml elution volume. If treated carefully, and washed scrupulously, a single calibrated column may be used for 20-50 determinations before requiring repacking and recalibration. The fact that a number of normal plasmas are necessarily included in the assay samples allows a continual check on the state of column calibration.

Plasma samples (1 ml) are applied to the column over a layer of 40% sucrose (0.5 ml) to sharpen the original boundary zone. Buffer is carefully layered over the column when the plasma layer has sunk just below the top gel layer and the buffer feed is then connected. Effluent samples (0.8 ml) are collected and assayed for fibrinogen by (a) thrombin-clottable protein⁶⁰ and/or (b) by radial immunodiffusion⁶¹ using specifically-absorbed fibrinogen antiserum. Effluent fibrinogen concentration is plotted against effluent volume and the plot compared with that of a normal plasma of identical fibrinogen concentration (a computer program which is now under development will be used to quantify this last step.)⁵⁹

This method of fibrinogen determination in its present form is exceptionally laborious to perform and is technically difficult. However, the applicant has several years experience using column chromatography methods and the principle or co-investigator plans to spend sufficient time in Dr. A. P. Fletcher's laboratory in St. Louis to develop the skills necessary to obtain satisfactory and reproducible results before attempting these studies in this research center.

(2) Isolation of plasma proteins

Preliminary studies will be conducted to determine whether or not the factor that increases platelet function and found in plasma of rats after smoking or nicotine treatment is also found in serum and urine of human volunteers and treated animals. Other preliminary studies will be conducted to determine heat and pH stability and whether or not the factor is dialysable. The purification techniques to be used on plasma from treated animals include: 1) column chromatography; 2) ethanol and $(\text{NH}_4)_2\text{SO}_4$ precipitation; 3) polyacrylamide gel electrophoresis; 4) gel filtration through Sephadex columns; 5) molecular sieving; and 6) recycling chromatography. In addition, density

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gradient ultracentrifugation has been of value in achieving partial separation of other plasma compounds and will be utilized in these studies in an attempt to partially purify the plasma factor. In this work assistance has been and will continue to be given by personnel at Beckman Instrument Co. and at the Oak Ridge National Laboratory.

(3) Platelet Production Studies

(a) Peripheral Platelet Counts

For platelet counts, blood will be taken from the leg vein of rats and diluted in ammonium oxalate to lyse the red blood cells. After 20 to 30 min of mixing on an automatic rotor, a drop of diluted blood will be allowed to flow under a cover slip onto a flat, thin hemocytometer chamber and allowed to settle for 20 min in a moist environment. Platelets will be counted under phase microscopy at 450 X by use of standard techniques.⁴⁵

(b) Platelet Life Span

Shortening of the platelet life span has been shown to be related to smoking in a study by Mustard and Murphy.¹⁵ Reduced life span indicates that the platelet is leaving circulation and either going into coagulation, into arterial walls or dying at a faster rate than normal platelets. If smoking shortens the life span of platelets, then one possible finding (although other hypotheses are possible) is that more young, active platelets will be in the circulation. Since younger platelets are larger and more functional than older platelets, this condition could help explain the increased rate of thrombosis among smokers. Recent unpublished results by the applicant indicate that nicotine ingestion does alter the life span of platelets.⁴⁶

Variations in platelet life spans will be determined according to the method described previously.⁴⁷ Rats exposed to either nicotine or tobacco smoke will be given daily isotopic injections of $\text{Na}_2^{35}\text{SO}_4$ (1 μCi $^{35}\text{S}/\text{gm}$ body wt.). The animals will be killed 24 hrs after the last injection and the ^{35}S incorporation will be determined by use of a Packard Tri-Carb liquid scintillation counter. Rats will be injected with 2 ml of a 1:10 dilution of heparin (heparin sodium, 1,000 IU/ml) intraperitoneally 10 min prior to killing. A standard volume of blood will be collected into EDTA from each rat and diluted with saline to obtain a better platelet yield. The blood-saline mixture of each rat will be divided into two 15 ml conical centrifuge tubes and the platelets will be separated by differential centrifugation at 5°C. After slow centrifugation of the blood (107 x g for 30 min), the PRP-saline will be removed and the tubes spun more rapidly (760 x g for 20 min) to obtain a platelet pellet.

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The platelets will be washed twice with 4 ml of saline and resuspended in 0.5 ml saline. WBC and platelet counts will be made on each platelet suspension. Two samples of platelet suspensions will be added to polyethylene counting vials each containing 10 ml of a scintillation mixture prepared in the following way: 1 liter of 1,4 dioxane; 175 gm of naphthalene; 7 gm of 2,5 diphenyloxazole; and 0.375 gm of 1,4-bis-2-(4 methyl-5 phenyloxazole)-benzene. The radioactivity of two samples of platelet-free plasma-saline and one sample of each wash will also be determined for control purposes.

If the platelet life span is found to be altered, one must determine if the alteration is due to the platelets themselves or to plasma factors. To make this determination, animals exposed to smoke and control animals will be given ^{35}S injections as described above. Platelets from both groups will be harvested along with platelet-free plasma. Animals exposed to smoke and control animals will be injected with the labeled platelets from the opposite group and the disappearance time of the labeled platelets from the plasma of each group will be recorded. If labeled control platelets injected into smoke-treated animals disappear from the circulation faster than labeled smoke-treated platelets in control animals, then the change in life span can probably be ascribed to some plasma factor alteration rather than to alteration of the platelets. Conversely, if labeled smoke-treated platelets injected into control animals are removed from the circulation faster than labeled-control platelets in smoke-treated animals, then a platelet alteration should be considered.

(c) Platelet Size

Platelets normally decrease in size with age through a process of maturation or senescence.^{48,49} Since younger platelets are larger than older platelets, the younger platelets could be more easily activated through contact with vessel walls and as a result lead to thrombus formation. Some studies have shown that the younger platelet is in fact, more functional than the older platelet.⁵⁰ The ability to determine increases in platelet size could possibly be used as a diagnostic tool in detecting potential heart attacks. If smoking causes an increase in the platelet size, then this could help explain the increase in platelet function which could help to potentiate the higher frequency of heart disorders among smokers.

Platelet size will be determined from washed platelet preparations by use of an Electrozone Celloscope attached to a 128 channel analyzer for cell sizing as in the Coulter counter method. Platelet suspensions for counting are made by diluting 5 μl of PRP into 10 ml Isoton (Coulter) to give a concentration of 1:2000. After the platelet suspensions have been counted, a value between 15,000-19,000 platelets (optimum number for sizing) will be used for sizing.

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(d) Megakaryocyte Determination

Since platelets are derived from the cytoplasm of megakaryocytes,⁵¹ one would expect relative changes in peripheral platelet numbers to be reflected by proportional changes in the megakaryocyte pool.⁵² If exposure of rats to tobacco smoke causes an altered platelet life span (as measured by ^{35}S incorporation) or increases their removal from circulation by some process (as determined by size changes), then one would expect the megakaryocyte population (or a precursor cell) to be stimulated. If smoking causes a reduction in the peripheral platelet count, then one would expect megakaryocytopoiesis to be stimulated by the action of thrombopoietin.

Bone marrow samples will be obtained from the excised tibias of rats by forcing air through the bone with a needle and syringe according to the method of Jackson.⁵³ Megakaryocytes constitute roughly 0.1% of the total bone marrow cell population and usually are not distributed evenly in smears or squashes. Bone marrow suspensions will be collected on Millipore filters with a 0.45 μ pore size. Marrow suspensions will be prepared by adding 2-3 drops of 3.5% polyvinylpyrrolidone in saline to the marrow cells on a glass slide. After the marrow suspensions and polyvinylpyrrolidone are mixed, then 20-40 microliters will be diluted in 5 ml of saline. Two ml of this latter suspension will be forced through a Millipore filter with a syringe adaptor. The filter will be removed and allowed to dry before staining the megakaryocytes. Microscopic analysis will be used to count the megakaryocytes.

(4) Chemical Alterations of Platelets

(a) Mucopolysaccharide (MPS) Content

It has been demonstrated that increased platelet adhesiveness is correlated with an increase in the MPS of platelets.⁴⁷ Recently, nicotine injections have been shown to increase the uptake of $\text{Na}_2^{35}\text{SO}_4$, indicating an increase in the amount of MPS.³⁷ There is conflicting evidence in the literature concerning smoking and platelet adhesiveness and since MPS content is known to alter platelet adhesiveness, it is possible that smoking may alter the MPS content of platelets. Therefore, MPS determinations on platelets exposed to nicotine or smoke will allow one to correlate any MPS changes with alterations in platelet adhesiveness. Hence, increased MPS would strongly indicate potentially increased adhesiveness which could account for the increased thrombotic tendency among smokers.

MPS determination will be made following the method outlined by McDonald.⁴⁷ Rat platelets will be labeled *in vivo* by daily intraperitoneal injections (1 μCi of $\text{Na}_2^{35}\text{SO}_4/\text{gm}$ body wt.) for 5 days before killing and determining the uptake of the isotope. At each time period the platelets of three rats will be pooled and resuspended into saline to a final volume of 2.5 ml. The radioactivity of 2 samples of the platelet suspensions, 2 samples of plasma-saline and 1 sample of each wash will be determined by use of a liquid scintillation counter as described above.

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To extract MPS from platelets, duplicate 1 ml samples of ^{35}S labeled platelets will be added to 1 M NaOH and hydrolyzed for 7 hrs at 5°C with constant mixing. The samples will be neutralized with 1 ml of 4 M acetic acid. The samples will then be treated 4 times with chloroform-Amyl-alcohol reagent to remove protein. After each treatment, the samples will be centrifuged ($760 \times g$ for 10 min) and the upper layer containing the MPS will be transferred to another tube. The final supernatant fluid will be dialyzed for 40 hrs against four 1,000 ml portions of distilled water at 5°C . After lyophilization, the material will be dissolved in 500 μl of water.

For identification of specific MPS molecules, samples of extracted ^{35}S -MPS will be subjected to ascending chromatography using MgCl_2 (0.5 M and 1.0 M), 0.4 M AlCl_3 or 0.5 M FeCl_3 as solvents. After chromatography the samples will be washed, dried and the strips cut into three pieces which will be placed in scintillation vials containing 10 ml scintillation fluid previously described and the ^{35}S activity counted by a liquid scintillation counter. On the basis of the chromatographic method described by McDonald⁴⁷ it will be possible to identify heparin and chondroitin sulfates A, B, and C.

(b) Hexosamine Determinations

Since hexosamines (glucosamine and galactosamine) are normally present as an MPS component, one would expect to find increases and decreases in hexosamines closely correlated with changes in MPS content. If smoking causes an increase in platelet MPS, then one would expect hexosamines to be increased also. Hexosamine determinations should, therefore, give more validity to the MPS determinations.

The determination of hexosamines will be done according to the following method:⁴⁷ rat blood platelets will be collected as previously described and resuspended in 1.2 ml of physiological saline. One ml of the platelet suspension will be added to 1 ml of 2 M HCl and placed in a hot water bath (100°C) for 4 hrs to break the glycosidic and sulfate bonds of the MPS complex. After heating, the samples will be allowed to cool and 1 ml of water will be added to each tube. One ml of freshly prepared acetyl-acetone (2,4 pentanedione) mixture (1 ml of acetylacetone in 50 ml of 1 M Na_2CO_3) will be added to the samples and 0.1 to 1.0 μmole of glucosamine or galactosamine. The platelet samples will again be placed in a hot water bath (100°C) for 10 min, cooled and 5 ml of 95% ethanol will be added to precipitate protein. Next, the samples will be placed in a water bath (75°C) for 5 min after which 1 ml of p-dimethylaminobenzaldehyde (specific stain for glucosamine and galactosamine) will be added and mixed thoroughly with a Vortex mixer. The tubes will then be placed back in the 75°C water bath for 30 min with occasional shaking in order to get maximum adsorption of benzaldehyde to hexosamine resulting in a color change. After removal and cooling, the optical density (OD) of the samples will be determined at 520 m μ . The OD readings will be compared to readings of known amounts of glucosamine and galactosamine and the amount of hexosamine can then be determined from a calibration curve of OD versus hexosamine concentration.

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(c) Protein Determination

Total protein determination of blood platelets will be used as a relative measure of size and therefore functional state. A younger and hence larger, more active platelet population should show more total protein per platelet.³⁸ If smoking or nicotine shortens the life span, then one would expect an increase in the total protein/platelet in the platelet population since more younger platelets will be in circulation.

A modified Lowry's method of protein determination will be employed.⁴⁷ One hundred microliters of platelet suspension will be added to 3.9 ml of water, mixed, and 0.5 ml of this suspension will be placed in a test tube. To each tube 5 ml of an alkaline solution (4% filtered Na_2CO_3 , 2% CuSO_4 , 4% sodium potassium tartrate 100:1:1 by volume) will be added and mixed well. The tubes will be placed in a 40°C water bath for 15 min after which 0.5 ml of Folin-Ciocalteu reagent diluted 1:2 with water will be added to each tube and mixed. After allowing 30 min for color development, the samples will be read at 660 mμ with a spectrophotometer. The OD readings for the samples will be compared to readings of known amounts of bovine serum albumin.

(5) Thrombopoietin Assays

It has been claimed that platelet life-spans are shortened due to smoking or nicotine treatment.¹⁵ In addition a decrease in peripheral platelet count has been claimed due to smoking.^{2,31} It seems possible that part of the increased function of platelets from individuals who smoke may be due to an increase in younger platelets in the peripheral blood made available by alterations in platelet production rates. Platelet production is thought to be controlled by a hormone (thrombopoietin) that is released and acts on immature megakaryocytes to increase the output of platelets in response to thrombocytopenia. Whether or not smoking causes an increase in serum thrombopoietin levels remains to be shown.

(a) Bioassay

Male C_3H mice weighing 22-25 g are used as assay recipients. The mice are injected intraperitoneally with rabbit anti-mouse platelet serum (AMPS) which is prepared and absorbed with mouse RBC as previously described.⁵⁴ Usually 0.1 ml AMPS is diluted to 0.5 ml in saline prior to injection. AMPS-injected mice with initial platelet counts (determined 4 hrs after AMPS injection by direct phase-contrast microscopy from a single drop of blood obtained by retroorbital puncture) above 50,000/mm³ are excluded from the assay. By use of this procedure, only 275 of 3,258 mice (8%) used in recent bioassays have been discarded. This strain of mice has a normal platelet count of about $9 \times 10^5/\text{mm}^3$. Mice are injected subcutaneously 4 times (2 times on days 5 and 6) with the first injection given 5 days after AMPS with test substance. Thirty μCi of $\text{Na}_2^{35}\text{SO}_4$ diluted in 0.5 ml of saline are injected intravenously on day 7 and the 24 hr radio-sulfate incorporation into platelets is determined. At the time of assay, mice are injected intraperitoneally with 0.5 ml of a heparin-Nembutal-saline solution (1.0 ml heparin, 1,000 U.S.P. units; 1.0 ml Nembutal, 50 mg; and 10 ml of saline) and platelet counts and WBC counts are made from a drop of blood obtained by retroorbital puncture. About 10 min later mice are bled from the heart into plastic syringes containing 1.0 ml of 1.0% disodium-ethylenediamine-tetraacetic acid (Na_2EDTA) in 0.7% saline. The blood of each mouse is expressed into a 12 x 75 mm plastic tube and mixed with an additional 1.0 ml of Na_2EDTA . Platelets are separated from the blood by slow centrifugation (30 min at 50 x g) to obtain a platelet-rich plasma layer which is transferred into another tube and then centrifuged at a more rapid rate (15 min at 360 x g) to obtain a platelet button. Platelets from each mouse are washed with 0.5 ml of 1% ammonium oxalate, then with 1.0 ml of saline, and resuspended in 0.35 ml of Isoton (Coulter). Two 100 μl samples of the

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platelet suspension are placed in plastic counting vials for the determination of radioactivity. Another sample of the suspension is used for the determination of the number of platelets in the washed platelet suspension. For platelet counting, the suspensions are further diluted (1 to 2,000) in Isoton and counted by use of an Electrozone/Celloscope (Particle Data, Inc.) with a log converter. The percent ^{35}S incorporation of the platelets is calculated:

% ^{35}S Incorporation =

$$\frac{\left(\frac{\text{CPM of platelet suspension}}{\text{Platelet count of suspension}} \right) \left(\frac{\text{Body weight in grams} \times 7\%}{\text{CPM INJECTED}} \right) \left(\frac{\text{Peripheral platelet count/ml}}{\text{CPM INJECTED}} \right)}{\text{CPM INJECTED}} \times 100$$

(b) Immunoassay

The immunoassay utilizes a hemagglutination inhibition technique as outlined in previously published articles.^{55, 56, 57} Our results indicate that TSF can be detected and quantified in sheep sera by use of this immunoassay. We also believe that this technique represents an inexpensive method for assay of TSF.

(6) Study of Platelets and Plasma Factors in Human Volunteers

Thirty ml of blood will be drawn into vacutainers containing EDTA from each volunteer and the platelets harvested as indicated above for rat platelets. Some of the platelets will be subjected to function studies. Other samples from this platelet suspension will be utilized for chemical studies as outlined above except $\text{Na}_2^{35}\text{SO}_4$ will not be used. Identification of MPS from unlabeled platelets will be performed as previously described.⁵⁸ The quantification is not as good as in the ^{35}S method, but is sufficiently sensitive to determine large differences. The hexosamine data, and other biochemical measurements, however, do not depend upon the use of radioactive labels. For platelet adhesiveness measurements, 2 or 3 ml of blood without an anticoagulant will be utilized. The plasma will be saved from the initial centrifugation for plasma factor studies.

The first studies will involve about 20 heavy, chronic smokers and an equal number of non-smokers to determine which, if any, of the above platelet functions or platelet chemicals are altered by smoking. The two groups will be paired for age, race, and sex. We will make every effort to screen the volunteers closely to insure that drugs known to affect some of these determinations (such as aspirin, and other agents known to inhibit ADP-induced aggregation; i.e. antihistamines, local anesthetics, antidepressants, tranquilizers, etc.) have not been used by the subjects for several days before obtaining the blood samples. In this regard, other factors known to affect platelet parameters (i.e. time after eating, diet, state of health, stress, illness, alcohol, occupation, genetic factors, and exposure to various

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physical factors) will be considered as far as possible. If the scatter in physical and chemical data is widespread, then it will be necessary to investigate the health and habits of each volunteer more closely. The clinical hematology group headed by Dr. S. Krauss and located in this research center will collaborate in such studies if the situation demands it. If the data are not widespread, many of the chemical or physical factors mentioned may not need to be considered in greater detail.

Based on results noted in the study of heavy smokers, acute experiments will be carried out using groups of volunteers in a fasting state to consider the effects of smoking on platelets following smoking of 2-4 cigarettes in about 2 hrs. Some of these volunteers will be non-smokers, others will be subjects who have smoked in the past while still others will be current smokers with different smoking habits. Control subjects (roughly matching the categories just mentioned) will also be utilized in this study. These control subjects will provide data on which to determine the effects of obtaining several aliquots of 30 ml of blood in the short time period contemplated. Blood samples will be taken before smoking, following smoking, and at intervals thereafter to establish the rates at which platelet function or chemical makeup are reversible when smoking is curtailed. We plan to analyze the platelet suspensions and plasma collected in these acute studies in the same manner as outlined above for the studies in chronic smokers. All studies using human subjects will be carried out in conformity with directives issued by our local committee on human welfare and experimentation to whom the protocol is being considered for approval. Appropriate forms for obtaining informed consent [i.e. (1) summary of explanation to patient for obtaining informed consent for experimental procedure; (2) consent for experimental procedure; and (3) forms required by The Council for Tobacco Research] of volunteers will be used. Copies of these forms are attached to this proposal.

(7) Phasing of Study

We plan to organize our research activities as follows: the first year will be devoted to animal studies designed to determine the platelet and/or plasma alterations, which are responsible for the hypercoagulability state found among smokers. Animal studies are specifically needed in order to maintain the highest level of control possible. In addition, experiments involving the use of isotopes are not possible in human subjects; therefore, certain platelet alterations such as production or exact MPS alterations necessitate animal models. If a plasma factor alteration is indicated, as our preliminary studies have shown, then quantification of several plasma proteins will be determined.

The second year will involve continued animal experimentation combined with human volunteers. These investigations will allow one to determine if certain changes in platelet activity of animals are analogous to those found in humans.

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The third year will be devoted to determining if the alterations in the platelets and/or plasma from animals exposed to smoke or nicotine are similar. In this terminal year we plan to screen human volunteers with the goal of demonstrating similar platelet and/or plasma alterations in humans who smoke as those found in animals where applicable. Some smokers have a significantly higher incidence of cardiac dysfunction as compared to the non-smoking population. It is hoped that the alterations found in these smoking volunteers will enable our group to diagnose a potential cardiac patient prior to the onset of the malfunction. Therefore, in order to determine a broad spectrum of platelet and/or plasma alterations found among smokers a combined study involving animal models and human volunteers needs to be undertaken.

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(90-26461-1A) 27.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Ample physical facilities are available for the principal investigator and co-investigator to conduct the proposed research project at the University of Tennessee Memorial Research Center and Hospital. These facilities include: adequate laboratory work space, a cold room, and a fully accredited animal facility. Although some of the equipment listed below is used by several programs, it is available on a part-time basis. These items include: a refrigerated centrifuge, a freezer, phase microscope, Model A Coulter counter, Model 3375 Tri-Carb Liquid Scintillation Counter, photographic equipment, analytical balance, and lyophilizer. In addition, a celloscope with a 128 channel analyzer, a Chrono-log aggregometer, and a Fisher Autocytometer are available. A new well-equipped medical library has been established in the Memorial Research Center. Excellent library facilities at the Oak Ridge Associated Universities and at the Oak Ridge National Laboratory are readily available.

Senior members of the staff of the Memorial Research Center are available for consultation and are currently utilizing several research methods which would be applicable to the studies proposed in this application. The members of the staff and their fields of interests are: Dr. R.D. Lange, erythropoietin and use of the HAI technique for detection of ESF; Drs. A.I. Chernoff, J.F. Fuhr, and D. Dupourque in amino acid analysis, identification of abnormal hemoglobins, and investigation on the effect of smoking on the stability, oxygen affinity, and binding of carcinogenic ligands to hemoglobin; Dr. W. Porter in glycoprotein structure; Drs. P.W. Wigler and W.R. Farkas in RNA enzyme chemistry; Dr. A.L. Kretchmar in stem cell repopulation and mathematical models of hematopoiesis; Dr. T.J. Yan in immunology; Dr. A. Solomon in immunoglobulin structure; Dr. B.B. Lozzio in RES function and lymphocyte stimulation by phytohemagglutination; Dr. C.B. Lozzio in cytogenetics; and Dr. S. Krauss in white blood cell function in disease. In addition, Dr. T.T. Odell, Jr. of the Biology Division of Oak Ridge National Laboratory is available for discussion of work on platelets and their functions.

11. Additional facilities required:

(NONE

12. Biographical sketches of investigator(s) and other professional personnel (append):

SEE ATTACHED

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

SEE ATTACHED

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(28-35^{36.} deleted)

14. First year budget:

A. Salaries (give names or state "to be recruited")

| | % time | Amount |
|---|--------|--------|
| Professional (give % time of investigator(s) even if no salary requested) | | |
| P. McDonald, Ph.D., Research Associate Prof. | 25 | 5,000 |
| Marshall E. Reese, Jr., Ph.D. Research Associate | 100 | 10,000 |

Technical

| | | |
|-----------------------------|-----|-------|
| Rose Clift, Technician | 100 | 7,000 |
| Ann Beardsley, Secretary | 10 | 595 |
| Bill Wolfenbarger, Lab Aide | 50 | 2,315 |

24,910

Fringe Benefits (12% of S&W)

2,989

Sub-Total for A

27,899

B. Consumable supplies (by major categories):

| | |
|---|-------|
| Animal Charges | 3,450 |
| Glassware, hardware, plasticware | 500 |
| Chemicals, syringes, disposable supplies and cigarettes | 900 |
| Isotopes | 500 |

Sub-Total for B

5,350

C. Other expenses (itemize):

| | |
|-------------------------------------|-----|
| Service contracts, maintenance | 800 |
| Computer time | 500 |
| Office supplies, xerox, photography | 400 |
| Publications and printing | 200 |
| Travel | 600 |

Sub-Total for C

2,500

Running Total of A + B + C

35,749

D. Permanent equipment (itemize):

| | |
|---|-------|
| Smoking machine | 2,000 |
| Updating existing instrument to dual channel aggregometer | 500 |
| Calculator (Marchant Model 730) | 300 |

Sub-Total for D

2,800

E. Indirect costs (15% of A+B+C)

5,362

Total request

43,911

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 29,573 | 5,850 | 3,000 | 300 | 5,763 | 44,486 |
| Year 3 | 31,347 | 6,350 | 3,500 | -0- | 6,180 | 47,377 |

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14. Explanation of Budget:

Consumable supplies:

Animal charges: Current animal charges for rats are: \$2.50 each purchase price and \$2.50/rat/month care. 600 rats will be needed the first year - \$3,000; 300 mice for TSF bioassays will be needed; each mouse costs \$1.00 and care is \$.50/2 months/mouse, \$450; $\text{Na}_2^{35}\text{SO}_4$ is used as a platelet label. This isotope costs \$50/5 mCi.

Computer time is calculated at rates currently available to this institution. Time includes program, consultation and running time.

Travel:

To send the principal investigator to one meeting and the co-investigator to two meetings dealing with platelet function and smoking.

Equipment:

A new smoking machine costing about \$2,000 is needed in this work. The machine that we are currently using is adequate for our preliminary studies, but several problems inherent to its design exist. First, smoke is forced through restricted openings which allow the build-up of coacervate smoke particles at an extremely rapid rate, thus decreasing the amount of smoke available for inhalation. Another portion of the standard puff (15-20%) is trapped in the cigarette holder before the smoke reaches the exposure chamber, again reducing the actual exposure. The most serious design problem with our machine is the need for immediate mixing of the fresh smoke with air which would greatly reduce coacervate formation as well as providing a consistent smoke-air mixture for inhalation. At present the animals are rotated systematically in order to insure a more even dose of smoke. Only 5 rats can be exposed at one time in our machine, the modified Walton will allow us to increase this number thereby increasing our sample size.

Updating the existing aggregometer to a dual channel instrument is needed in order to measure the large number of platelet samples planned. The dual channel updating on the aggregometer will allow us to aggregate the samples more rapidly as well as allowing for larger sample sizes. As Levine²³ recently indicated, aggregations need to be performed within 30 min after bleeding. The dual channel attachment will greatly reduce the lag time between aggregations.

Salaries:

For years 2 and 3 the salaries are computed at 6% increase per year.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--|--------|--------------------|
| Platelet Function and Smoking | American Medical Association Education Research Foundation | 87,097 | 5/1/71 to 4/30/74 |
| Characterization and Immunoassay of Thrombopoietin | American Heart Association | 53,680 | 7/1/72 to 6/30/75 |
| Production, Purification and Assay of Thrombopoietin | U.S. Atomic Energy Commission | 33,000 | 6/1/73 to 5/30/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|---|---------|--------------------|
| Thrombopoietin: Immunoassay and Characterization | National Institute of Health Approved but not funded #HL-14637-01A2 | 121,993 | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept to Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University of Tennessee

Mailing address for checks

University of Tennessee

Cumberland Avenue

Knoxville, Tennessee 37916

Principal investigator

Typed Name Ted P. McDonald, Ph.D.

Signature *Ted P. McDonald* Date 12-14-73

Telephone (615) 971-3748

Area Code Number Extension

Responsible officer of institution:

Typed Name Hilton A. Smith

Title Vice Chancellor for Grad. Studies & Res.

Signature *Hilton A. Smith* Date 1/1/74

Telephone (615) 974-3466

Area Code Number Extension

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#519C - SOLORP

1003540274

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 12, 1974

Grant application No. 519C

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson and Liebow

Subject: Louis A. Soloff, M.D., Temple University, Philadelphia
Continuation application No. 519C
"Role of lecithin: cholesterol acyltransferase (LCAT) in cholesterol metabolism in health, disease and during smoking"

History

Grant No. 519 has been funded by CTR since 1966. A predecessor grant, No. 374, was funded from 1963 - 1966.

Grant No. 519BRL "Purification and physiologic significance of lecithin cholesterol acyl transferase (LCAT)", now in effect in the amount of \$62,500 a year, was awarded without assurance of continued support.

Therefore the enclosed request competes without commitment.

Request

Application No. 519C requests \$75,873 plus two additional years.

Document Submitted

Enclosed is application dated 1/21/74.

Comment

Dr. Bing visited Dr. Soloff October 19, 1972.

On March 27, 1973 Drs. Soloff and Lacko saw Dr. Gardner at the CTR offices.

Re-orientation of the study, resulting in part from the above discussions, is evident both in the text and in the new title.

FWN:gh

Encls.

FWN
F.W.N.

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ack
1/29/74
#5196

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/21/74

JAN 29 1974

1. Principal Investigator (give title and degrees):
Louis A. Soloff, M.D.
Blanche P. Levy, Distinguished Service Professor of the University; Professor of Medicine; Director, Research Lipid Laboratory; Member, Research Advisory and Review Committee for the Clinical Research Center.
2. Institution & address:
Temple University Health Sciences Center
3401 North Broad Street
Philadelphia, Pennsylvania 19140
3. Department(s) where research will be done or collaboration provided:
Research Lipid Laboratory
Division of Cardiology
Department of Medicine
Temple University Health Sciences Center
4. Short title of study:
Role of lecithin:cholesterol acyltransferase (LCAT) in cholesterol metabolism in health, disease and during smoking.
5. Proposed starting date: July 1, 1974
6. Estimated time to complete: Three years.
7. Brief description of specific research aims:

LCAT is an essential factor in plasma cholesterol esterification. For this reason, a study of this enzyme should increase our overall knowledge of lipid metabolism. There is already evidence that patients with LCAT deficiency develop atherosclerosis even though their levels of blood cholesterol and beta-lipoproteins are far below normal. It therefore appears worthwhile to determine whether LCAT plays a role in other patients with atherosclerosis and if so, possibly to add another method of separating genetic from environmental factors. So called risk factors, including smoking and resistance to these risk factors cannot be evaluated if genetic factors are ignored. Purified LCAT appears essential to supply more complete information on the role of LCAT in lipid metabolism in health and disease. We already have made a preparation of human LCAT which is stable and 4000 to 10,000 times as concentrated as that present in human plasma. We aim 1) to produce LCAT antibodies for isolation and quantification of pure LCAT 2) to determine LCAT's physical, chemical and enzymatic properties 3) to determine LCAT activity during physical, pharmacologic and hormonal stimuli and during nicotine administration and smoking in the experimental animal, in an invitro system containing plasma with and without LCAT and red blood cells, and arterial wall red blood cell plasma with and without LCAT and in humans with and without obstructive coronary heart disease documented by coronary arteriography

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8. Brief statement of working hypothesis:

Glomset¹ postulated that plasma lipoproteins pick up cholesterol from a variety of cells by a mechanism involving equilibrium between lipoprotein bound and cell free cholesterol. LCAT is postulated to convert free cholesterol to cholesterol esters which are removed from plasma, hydrolyzed by the liver and transformed into bile. As a result of plasma esterification, the equilibrium may be shifted so as to allow egress of cholesterol from cells. More recently, Glomset and Norum² have postulated an 180° opposite mechanism, namely, that LCAT functions to maintain a normal content of cell membrane cholesterol. In either event the result might be an alteration in cell surface which prediposes it to the accumulation of cholesterol. That LCAT might play a role in atherosclerosis is suggested by 1) our findings that LCAT activity is inversely related to species susceptibility to atherosclerosis (manuscript³), 2) that an atherogenic diet inhibits cholesterol esterification⁴, 3) our findings that the fractional rate of cholesterol esterification of cholesterol is abnormal in familial hyperbetalipoproteinemia⁵, 4) Gjone's demonstration of severe atherosclerosis at autopsy in a 40 year old woman who died with LCAT deficiency and a blood cholesterol of only 60mg/dl. and his findings of atherosclerosis in other patients with LCAT deficiency. He writes "Why these patients with this lack of plasma cholesterol esters and low levels of apolipoprotein B develop atherosclerosis remains to be explained"⁷, and 5) evidence that high density lipoprotein apoprotein, poor in surface cholesterol and with a high sphingomyelin/lecithin ration, postulated effects of LCAT, is most capable of removing membrane cholesterol⁸. Smoking LCAT interrelations will be studied, particularly in atherosclerotic patients.

9. Details of experimental design and procedures:

We intend to seek answers to these problems in several different ways based upon our past work on LCAT and on work done by others. A summary of our work on LCAT was presented at a private international symposium on LCAT in October, 1973 in Oslo, Norway. This symposium including our manuscript⁹, a copy of which is inclosed, will be published in April, 1974 as a supplement to the Scandinavian Journal of Laboratory and Clinical Investigation. So far as we know, we are the only ones who has prepared a stable preparation of LCAT which is 4,000 to 10,000 times the concentration present in normal human plasma.

At the present time we are testing the activity of this preparation in an invitro system containing apolipoprotein glutamine I and II, the putative natural activator and inhibitor of this enzyme.

However, there are other possible activators and inhibitors as shown in our work with heparin¹⁰ (manuscript enclosed accepted for publication by Biochimica et Biophysica Acta). The electron morphologic changes that occur in such studies are being studied by Dr. Hisashi Shinozuka, Associate Professor of Pathology and member and electronmicropathologist, Fels Research Institute of our Medical School. These electronmicroscopic observations will test Shumaker and Adams's hypothesis that LCAT has as one of its functions the maintenance of lipoprotein structure as triglycerides are removed from very low density lipoproteins.

However, because of the possibility of other as yet unknown inhibitors and activators of LCAT activity, because of the small amount of the enzyme present in the plasma and also because of the complex interaction between the enzyme and its substrates we think it is imperative to develop a method that assures purity of the enzyme and is capable of measuring accurately minute amount of the enzyme in the range found in the plasma and of the very minute changes that might occur in different states. For this reason, we have chosen an immunologic approach in an attempt to solve this problem.

(see following pages)

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Radioimmuno assay of LCAT

Methods of procedure:

1. Production and characterization of antibody to LCAT

a) Purification of antigen (LCAT):

Our laboratory is one of two in which highly purified LCAT has been obtained. We have worked out a procedure for preparing greater than 4,000 fold purified LCAT which is stable for at least eight hours, with a yield of 5%.

A litre of outdated plasma is first mixed with 20 ml of 10% egg lecithin suspension and 1 ml of mercaptoethanol and 440 gms of ammonium sulfate. The precipitate after collection by centrifugation is dissolved in 0.005 EDTA pH 7.4 and its density is adjusted to 1.22 with cesium chloride. The LCAT is layered under CsCl of $d=1.21$ and floated by centrifugation at 100,000 g. for 36 hours. LCAT bands at a density of 1.20 where most of the lipoproteins float at $d=1.18$ and most of the plasma proteins settle at the bottom at 1.26. The clear zone between the plasma proteins and lipoproteins is dialysed and fractionated on a DE-52 column using tris-EDTA-NaCl pH 7.4 and a sodium chloride gradient. The eluted enzyme is then put on a HDL-sepharose column and washed with tris-EDTA-NaCl buffer. After the protein peak has come out the enzyme is eluted with 0.05 tris-0.4M glycine buffer pH 9.0. The protein was estimated in all these steps by the fluoroscamine method¹¹ after dialysis. About 500 ug of enzyme is obtained with a 5% yield. On standard polyacrylamide gel electrophoresis two bands are seen and the enzyme activity is associated closely with the fast moving band.

On immunoelectrophoresis with hyperimmune equine serum produced against whole human serum (copy of photograph enclosed) a single arc is the only one obtained. We are currently affinity labelling the enzyme with radioactive diisopropyl fluorophosphate to identify the enzyme bands. Since those obtained on immunoelectrophoresis are close to that of HDL and albumin the possibility that albumin and/or HDL is contaminating the enzyme exists. These are currently under study.

b) Production of antibody.

100 ug of purified LCAT will be administered into young rabbits intravenously in 0.1 to 0.2 ml of saline. On the fifth day the animals would receive 100 ug of the enzyme with complete adjuvant (1:L) intramuscularly. Weekly intramuscular injections of 100 ug will be given thereafter. The animals will be bled weekly and their antibody production will be checked by crossed immunoelectrophoresis¹². Once high antibody titer is obtained by immunoelectrophoresis (a method of higher resolving power) the enzyme and antisera will be carried out to check for the presence of possible contaminants in the enzyme preparation and their antibodies in antisera.

c) Characterization of antibody.

If contaminating antibodies are found in the antisera, they will be first identified by using pure plasma proteins (albumin, HDL etc) and then absorbed out. In order to purify and enrich LCAT antibody, the antiserum, after removing the contaminating antibody with the pure contaminating antigen will be put on a sepharose-LCAT column. After eluting non antibody proteins the LCAT antibody will be eluted with a gradient of guanidine-HCl as suggested by Weintraub and Kadesky¹³. The fractions will be

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assayed by radioimmuno assay (see below) and those showing the highest titre will be used as purified antibody.

For purposes of establishing absolute identity of anti LCAT¹ antibody, DFPH³ labelled LCAT will be used as antigen and precipitates obtained by the double antibody technique of Weintraube and Rose¹⁴. The formation of precipitates containing H³ label will be considered as indicative of the presence of antibody to LCAT. For the further characterization electrophoretic and chromatographic techniques using DFPH³-LCAT incubated with antiserum will be used¹⁵.

Radioimmuno assay: The highly purified preparation obtained after affinity chromatography will be concentrated by vacuum dialysis to give .5 to 1 mg/ml enzyme solution. After adjusting pH to 7.6 with 1.0 M monosodium phosphate the sample will be injected into bottles containing carrier free I¹²⁵ of 1-5 millicurie. 216.0 mg. of chloramine T and 0.3 mg of potassium iodide will be added and the reaction mixture will be incubated for 30 minutes at room temperature. The reaction will be stopped by adding sodium bisulfite. The unreacted iodine will be removed by sephadex chromatography using G-25. A protocol for the assay is given:

| | |
|-------------------------------------|----------|
| Unlabelled enzyme or serum | 0.5 ml * |
| Labelled enzyme diluted | 0.1 ml * |
| to contain approximately 2-10 ug | |
| Antibody - diluted rabbit antiserum | 0.1 |
| First incubation | 1-5 days |
| Antibody to rabbit globulin | 0.1 ml |
| Incubation | 48 hrs. |

* unlabelled:labelled to vary from 0.9 to 0.1 (w/w)

The assay will be performed in equivalent zone and the condition of the assay system like the dilution of LCAT antiserum, the antiserum to rabbit γ globulin, period of incubation etc will be determined using the standard of known protein concentration and known iodine labelled enzyme. The precipitated I¹²⁵ after 2nd incubation will be collected by centrifugation and the counts in the precipitate and supernatant will be determined. The dose response curve will be constructed after logit transformation as suggested by Rodbard¹⁶ and the following statistics will be evaluated on a sequential and cumulative basis using wang-300 programmable calculator.

- % radioactivity bound in the absence of unlabelled enzyme %B
- Slope of the linear position of %B vs log dose curve
- 50% intercept
- within assay standard deviation
- between assay standard deviation

Effect of physicochemical factors on the assay:

Since the enzyme has been proposed to exist as a complex with HDL¹⁷ the effect of pH 9.0 and taurocholate, agents known to dissociate LCAT from HDL, will be studied on the radioimmuno assay. At pH 9.0 the antigen-antibody complex formation will not be expected to be seriously affected. The effect of agents that inhibit LCAT such as sulphhydryl blockers, reagents, polyvalent cations will also be studied on the antigen antibody reaction.

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Application to Plasma:

From the yield of purified enzyme obtained by Fielding and Fielding¹⁸ and in our laboratory the concentration of LCAT in plasma may be calculated to be approximately 0.1-0.5 mg%. Hence each assay system will contain about 0.1 ug of labeled enzyme in 0.1 ml and plasma diluted to give approximately 0.01 to 0.9 ug LCAT (two times to 100 times). After carrying out the incubation as mentioned in the protocol the labell in the precipitate and supernatant will be determined. It should be possible to carry out about 100 samples with each labelled batch of enzyme. The purified enzyme will be used as a standard, defined arbitrarily as 1 unit/ug of protein. For studies on pathological serum 6-10 normals will be used as separate controls. The values obtained will be compared with the enzyme activity determined by GLC.

Animal Studies:

Since the ultimate goal of the research project is to evaluate the role of LCAT in the homeostasis of plasma cholesterol and in atherosclerosis, we plan to study the effect of LCAT infusion in experimental animals and its effect on plasma cholesterol metabolism. Rabbits and guinea pigs are selected for this purpose since the former has been extensively used as an experimental model for atherosclerosis and the latter has been shown to develop lipoprotein abnormalities similar to that in LCAT deficient humans, when these animals are fed cholesterol. However repeated infusions of LCAT may produce immunological reactions. These will be circumvented by the use of antilymphocyte serum.

Experiments with rabbits:

A group of 20 rabbits will be divided into groups of eight and 12 and the latter will be fed cholesterol containing diet for 20 days. These will be divided into three groups 1) four animals left untreated 2) four animals treated with antilymphocyte serum (ALS) 3) four animals treated with ALS and LCAT. Those on normal diet will be divided into two groups of four and one will be treated with ALS. Concentrated purified LCAT will be administered intravenously every third day and ALS will be administered once a week. Before and after the experimental period (15 days) the following will be compared in all five groups: cholesterol levels of plasma (free and ester) and presence geographically of atherosclerotic lesions and if necessary their lipid composition.

Guinea Pigs:

Similar experiments will be carried out in guinea pigs but in addition to determining serum cholesterol levels and the presence of atherosclerosis, the lipoproteins will be analyzed for abnormal LDL and HDL using gel exclusion chromatography and electron microscopy. The effect of LCAT treatment on plasma cholesterol levels in these animals, on atherosclerotic plaques in the rabbit and on the abnormal lipoproteins of guinea pigs will be determined.

While attempting to develop radio immuno assay of LCAT, we are evaluating two assay systems for their respective efficiency and applicability to the routine measurement of the initial rate of esterification of cholesterol in human serum. We are comparing our modification of the Stokke and Norum method with a GLC method introduced by Marcel et al¹⁹. Their values are lower than ours. Furthermore their method has a relatively large error ($\pm 8\%$) and was performed on too few subjects to allow for firm conclusions on the relative merit of their technique.

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The role of erythrocytes in cholesterol metabolism:

Preliminary experiments in our laboratory have shown that erythrocyte cholesterol is probably esterified at a faster rate than plasma cholesterol.

TABLE 1

12 ml of fresh or heated serum (30 minutes at 56°C) were incubated with or without 3 ml. of erythrocytes. The amounts of free and total cholesterol were estimated at 0 and 6 hours.

| Chol. mg. | Fresh Serum | | Heated Serum | | Fresh Serum & Erythrocytes | | Heated Serum & Erythrocytes | |
|--------------|-------------|-------|--------------|-------|----------------------------|-------|-----------------------------|-------|
| | 0 | 6 hrs | 0 | 6 hrs | 0 | 6 hrs | 0 | 6 hrs |
| Total | 15.6 | 15.7 | 15.6 | 15.6 | 15.6 | 16.4 | 15.6 | 16.7 |
| Free | 4.8 | 3.4 | 4.8 | 4.6 | 4.8 | 3.4 | 4.8 | 4.7 |
| Ester | 10.8 | 12.3 | | | 10.8 | 13.0 | 10.8 | 12 |
| ΔEster | 1.5 | | 0.2 | | | 2.2 | | 1.2 |
| Erythrocytes | | | | | | | | |
| Free | 14 | | | | 1.6 | 0.59 | 1.16 | 0.55 |
| ΔFree | | | | | | 0.57 | | 0.61 |

The presence of erythrocyte in both cases (fresh and heat inactivated plasma) causes substantial increase in the extent of cholesterol esterification. This may be due to: 1) erythrocytes containing LCAT 2) Erythrocyte cholesterol is a better substrate than plasma.

It has been shown in the rat that erythrocytes have only 2% of plasma LCAT activity. Also, D'Hollander and Chevallier²⁰ injected rats with C¹⁴ cholesterol and studied the exchange of cholesterol between plasma and erythrocytes in relation to cholesterol esterification. They concluded that erythrocyte cholesterol was esterified before it mixed with the free cholesterol of plasma. They also observed the rate of esterification of cholesterol in blood to be much higher than that of plasma alone. We have analyzed the data of Hagerman and Gold²¹ on the exchange of C¹⁴ cholesterol between plasma and erythrocytes in the case of dogs in vitro, and have computed the LCAT activity from their data using plasma and erythrocyte cholesterol as substrate (Table II). It is clear that if plasma cholesterol is the substrate the computed LCAT activity would be too high a value compared to experimentally obtained plasma LCAT activity.

TABLE II Rate of cholesterol esterification during exchange of plasma and erythrocytes when one of the component is labelled.

| Assumed Substrate | Rate of Esterification/gm of blood/hr. | |
|-----------------------------|--|-----------|
| | Rat (a) | Dog (b) |
| Plasma | 120 ug | 180 ug |
| Erythrocyte | 70 ug | 40 ug |
| Observed rate other methods | 50 ug (c) | 40 ug (c) |

(a) reference #20

(b) reference #21

(c) our laboratory estimation

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The following experiments are designed to confirm our preliminary findings in humans using more sensitive methods and to further elucidate the nature of the cholesterol pools of the erythrocyte. In view of the total and rapid exchange of cholesterol between plasma and erythrocyte in vivo and the lack of the same under in vitro conditions separate pools may exist in erythrocytes of which only one may function as substrate for LCAT in vitro. We postulated that transit of erythrocytes through the capillaries causes a redistribution and homogenization of erythrocyte pools. Or, erythrocytes depleted of their cholesterol by the action of LCAT may regain their cholesterol complement by physical contact with the membranes of blood vessels, plasma membranes of liver, kidney, etc. These will be approached by in vivo experiments using rat as an experimental animal.

Methods of Approach

Blood from fasting normal human subjects will be collected in EDTA coated tubes. For the determination of plasma free and total cholesterol 2 ml. of aliquots of blood will be chilled in ice and the erythrocytes will be separated by centrifugation at 1000g for 10 minutes. At 4°C the erythrocytes will be washed four times with saline (0.9% and pH 7.3). The washings will be pooled with plasma and lipids will be extracted with 40 volumes of chloroform-methanol (2:1). A known aliquot will be dried under N₂. The erythrocyte lipids will be extracted with 80 volumes 2:1 chloroform-isopropanol and an aliquot will be dried under N₂. Total and free cholesterol will be determined by gas liquid chromatography techniques²¹ using automatic sample injector and electronic integration attachment. Five cholestane will be used as an internal standard. Changes in free cholesterol will be considered as equal to increase in esterified cholesterol.

For determination of specific radioactivity 10 to 20 ug of total cholesterol will be spotted on TLC plates and developed using petroleum ether; ether:acetic acid (80:15:2) as solvent. The cholesterol and ester spots will be extracted with acetone. An aliquot will be used for estimation of amount of cholesterol by GLC and another for radioactivity.

For invitro radioactive labelling of plasma H³ or C¹⁴, cholesterol in acetone will be slowly added to 5% albumin to make an emulsion which will be added to plasma (0.1 V plasma) for in vivo experiments with rats' blood (cholesterol will be labelled) by injecting H³ meralon and blood drawn by cardiac puncture. Administration of this labelled RBC will be done through the tail vein.

Approaches: 1) To determine the effect of erythrocyte on plasma LCAT activity blood and plasma will be incubated separately for 0.5, 1, 2, 4, 8, and 24 hours. Free and total cholesterol of plasma and erythrocytes will be determined for each period. Change in free cholesterol would be considered as equal to the cholesterol ester formed. Blood would be expected to show more of the esterification compared to that of plasma. Also, the esterification loss of erythrocyte cholesterol would reach a plateau at about eight hours.

2) To find out whether erythrocyte cholesterol is a better substrate. C¹⁴ labelled plasma will be incubated for one hour at 37°C. At this time called 0 time red cells will be mixed to give a hematocrit of 40%. After incubation for 0.5, 1, 2, and 12 hours at 37°C specific activity and amounts of free and total ester cholesterol will be determined. A control without added red cells will be carried out throughout the procedure. Data will be analyzed as follows.

Rate of esterification for time period 't' =
$$\frac{\text{change in total label in cholesterol ester for time period 't'}}{\text{average specific activity of cholesterol substrate for the time period}}$$

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The rate of esterification computed using plasma and erythrocyte cholesterol average specific activities will be compared with the control plasma's rate. It is expected that if erythrocyte is really the substrate for LCAT, the ratio of esterification rate using specific activity of erythrocyte cholesterol to the control's rate of esterification will be closer to unity. When plasma specific activity is used to determine the rate of esterification in blood the ratio will be far less than 1, especially in the early period of incubation since the exchange has proceeded only very little and the specific activity of plasma cholesterol will be high (compared to that of red cells).

3) To test whether a specific pool cholesterol on red cells is the substrate for LCAT, exchange between plasma and erythrocyte cholesterol has been reported to be completed in 8-12 hours in vitro. Such red cells take up cholesterol when incubated with heated plasma. Thus the cholesterol lost through LCAT action could be replenished by labelled H^3 cholesterol if red cells are incubated in the heated plasma labelled with H^3 cholesterol. The 12 hour incubated mixture of experiment 22 will therefore be reincubated in heated plasma labelled with H^3 cholesterol for six hours. Samples will be incubated using this doubly labelled erythrocyte and unlabelled fresh plasma. The ratio of C^{14} to H^3 specific activity of cholesterol ester at different periods of incubation will be determined. This will be unity if there is only one pool for LCAT to act. On the other hand if LCAT substrate of erythrocyte was coming from a separate pool which is relabelled with H^3 , the ratio will be much less than unity. These experiments will be repeated with at least 10 different blood samples and in all cases student 't' tests will be performed when controls are compared with experimental samples. These three experiments will be repeated with rat blood. In addition plasma and red blood cells will be labelled in vivo by intravenously injecting them with C^{14} mevalonate. Experiments using labelled (in vivo) plasma and unlabelled rat red cells and vice versa along the lines used in experiments will be done. The calculations and analysis of data will be the same.

4) To determine whether factors in circulation are involved in the control of red cell cholesterol pools. Blood will be pooled from six rats and separated into plasma and cells. C^{14} labelled blood will be incubated at $37^\circ C$ for six hours to deplete the cell cholesterol. These cells will be infused into six rats through the tail vein. After 30 minutes, blood will be collected and cells will be separated. C^{14} of these cells will be determined. An aliquot of the labelled cells which have been preincubated with plasma for six hours will be mixed with unlabelled red cells to give the same C^{14} counts per ml of packed cells as that found for infused cells. These two kinds (one mixed in vitro with unlabelled red cells and the other mixed in vivo) of red cells will be incubated with unlabelled plasma and the rate of cholesterol ester C^{14} formation will be compared. If factors in circulation affected LCAT substrate of red cell an increased efficiency (more C^{14} in ester) will be noted in the case of infused cells.

Once the role of RBC as a substrate for LCAT reaction has been established and quantified, we shall return to the study of the interaction of arterial wall, RBC and plasma with and without LCAT activity. Preliminary studies in our laboratory suggest that these interactions have an important effect on intravascular and vascular cholesterol metabolism.

Interactions of human serum lipoproteins and LCAT with platelets:

We have observed that LDL strongly potentiated ADP induced platelet aggregation

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in suspensions of washed human platelets. On the other hand VLDL inhibited platelet aggregation. LDL did not cause platelet aggregation in the absence of ADP.

These observations have stimulated the following studies primarily proposed by Stefan Niewiarowski, M.D., Ph.D., who is Research Professor of Medicine and member of the specialized sector for Thrombosis Research at our Medical School. The lipid studies will be done in our laboratory and the hematological studies will be done in Dr. Niewiarowski's laboratory.

a) Study on the effects of isolated serum lipoproteins on the aggregation and release reaction of human platelets; b) the interrelationship between plasma and platelet lipoproteins; c) effects of platelets and platelet components on the activity of lipoprotein lipase (LP) and lecithin-cholesterol acetyltransferase. These three parts of our project will be discussed separately.

a) Lipoprotein will be prepared by the method of Havel, et al²³ as modified by Hatch and Lees²⁴. This method is based on the sequential flotation of lipoproteins by preparative ultracentrifugation. The lipoproteins are floated by increasing the density of each step by the addition of sodium bromide to the medium. The lipoproteins fractions are further purified by refloating them twice at the following densities: very low density lipoproteins (VLDL) at $D=1.06$; low density lipoproteins (LD) at 1.062 ; and high density lipoproteins (HDL) at 1.21 . Further purification of lipoproteins if necessary will be achieved by gel filtration using sephadex G-200, sepharose or biogel columns. Since free fatty acids may cause platelet aggregation, the free fatty acid content of the purified lipoproteins will be determined by the method of Dole and Meinert²⁵. Homogeneity of lipoproteins will be checked by electrophoresis and immunodiffusion. Platelets will be prepared from human blood, collected in blood bank on acid citrate dextrose anticoagulant, by means of the method Mustard, et al²⁶. In this method apyrase is used to prevent platelet aggregation during centrifugation. After washing, platelets are resuspended in Tyrode-albumin solution. These platelets are stable for at least three hours at 37°C , they are sensitive to low concentration of ADP, collagen and thrombin. Their ultrastructure and shape are preserved. ADP causes platelet aggregation in this suspension but it does not cause any significant release of platelet constituents, Thrombin and collagen cause both aggregation and release. Sensitivity of washed platelet to epinephrine is usually decreased. Washed platelets will be labeled with ^3H serotonin²⁷ for the purpose of study the platelet release reaction.

In typical experiments lipoprotein fractions will be incubated with the platelet suspensions before studying platelet aggregation induced by the four stimuli (ADP, collagen, thrombin, epinephrine) in the Payton aggregometer. After aggregation, platelets will be centrifuged in the Eppendorf centrifuge and the supernatant will be tested for the presence of the released platelet constituents such as ^3H serotonin, adenine nucleotides and platelet factor 4^{24} . The increased availability of platelet factor 3 during platelet aggregation and the effect of lipoproteins on this reaction will be tested. Platelet factor 3 activity will be evaluated by means of Russell's viper venom following the procedure described by several investigators²⁸. The effect of various inhibitors of platelet aggregation such as dipyridamole derivatives, aspirin, prostaglandin E_1 on the potentiation of platelet aggregation by lipoproteins will be studied.

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In another set of experiments, lipoproteins will be prepared from serum of patients with various types of hyperlipoproteinemia. The effect of these fractions on platelet aggregation and the platelet release reaction will be studied. Similarly, platelets will be prepared from the blood of patients with hyperlipoproteinemia and their sensitivity to various aggregating agents will be investigated.

Finally washed platelets will be suspended in normal plasma and in plasma of patients with abetalipoproteinemia. Aggregation of these platelets in the presence of increasing concentration of added LDL will be studied. Similar experiments will be performed using platelets isolated from the blood of patients with hyperlipoproteinemia and abetalipoproteinemia.

b) The interrelationship between plasma lipoproteins and platelet lipoproteins is not known. Studies in various laboratories are being carried out on the exchange between platelet and plasma lipids. We would like to determine whether platelet and plasma betalipoproteins have common antigens such as those belonging to the Lp or Ag system. The antisera will be prepared and antigen typing performed according the methods of Berg²⁹. If platelets and plasma share common lipoprotein antigens, it is more likely that changes in the level of plasma betalipoprotein will affect platelet function.

c) As it has been mentioned previously there are controversial data regarding the effect of platelets on lipoprotein lipase (LPL). Some authors describe platelets as containing an inhibitor of lipoprotein lipase^{30,31} while Bucher³² observed that platelet rich plasma contains more LPL activity than platelet poor plasma. The latter suggested that platelets may carry lipolytic activity absorbed on their surface. Soloff and Rutenberg found no effect on platelets on postheparin lipolytic activity. The discrepancy between the results of various authors may be related to the different experimental system used; the authors reporting an inhibition of LPL by platelets used an assay based on changes in optical density while Bucher used an assay based on the measurement of released free fatty acids. In our experiments we intend to compare both methods. Since platelets may absorb LPL as well as inhibit it partially, LPL activity will be assayed in four systems:

- a) "post heparin" platelet poor plasma; b) "postheparin" platelet rich plasma;
- c) "post heparin" platelet poor plasma and normal platelets; d) normal platelet poor plasma and platelets obtained from "post heparin" subjects.

The effect of platelet extracts and the material released during platelet aggregation on the activity of LPL also will be investigated.

Clearance of postprandial hyperlipaemia by heparin induced through activation of lipoprotein lipase, leads to an increase in the level of free fatty acids in serum. Whether this phenomenon may affect platelet function will be studied. Platelet aggregation in platelet rich plasma induced by ADP and other stimuli will be compared in four groups of individuals - a) fasting control; b) alimentary hyperlipaemia; c) fasting control injected with heparin; d) alimentary hyperlipaemia injected with heparin. Heparin is known to clear alimentary hyperlipaemia by release of fatty acids from chylomicrons and fatty acids are known to cause platelet aggregation³⁴. Therefore it is possible that individuals on non restricted diets may have increased platelet aggregation after heparin injection.

Lecithin:cholesterol acyltransferase (LCAT) is the enzyme responsible for the conversion of free cholesterol to cholesterol ester in human plasma. It is our intention to examine whether cholesterol transfer between platelets and plasma is promoted by the LCAT reaction, and to investigate the effects of platelets on the LCAT reaction.

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Cholesterol esterification will be measured by determining the amount of radioactive cholesterol that is esterified during the incubation period in the presence and absence of platelets. The labeled cholesterol will be added to plasma (which contains no active LCAT) as a serum albumin emulsion³¹. The reaction will be started by adding purified LCAT preparations to the radioactively labeled inactivated plasma. Esterification will be allowed to proceed to give linear rates over the selected time periods. Platelets will be isolated from human blood by the method of Mustard et al²⁶.

Interactions between Smoking and LCAT:

So called "risk factors" including smoking cannot be evaluated if genetic factors are ignored. Thus, homozygous familial hyperbetalipoproteinemic subjects have the highest incidence of coronary heart disease and at the youngest age whether they smoke or not. Statistical association can be misinterpreted as causal if there were still other as yet unknown genetic factors that make one more or less susceptible to coronary heart disease and/or if the genetic factors in some way predispose to smoking. Our ultimate aim is to determine whether LCAT activity which plays an important role in intravascular cholesterol metabolism is different in subjects with hypercholesteremia and in subjects with coronary heart disease than in healthy subjects and if so, whether such abnormalities are congenital or acquired and if they are influenced by smoking.

We have already shown that the initial fractional rate of serum cholesterol esterification is subnormal in most patients with myocardial infarction and in subjects with familial hyperbetalipoproteinemia. We now have similar findings in over 100 smoking and non smoking patients with coronary heart disease documented by coronary arteriography. Smoking appears to play no role either in the development of coronary heart disease or in LCAT activity judged by the coronary arteriographic studies. However, an interpretation of our statistical analysis is complicated by the fact that our patients had been on various drugs for their heart disease at the time they were catheterized.

We are therefore embarking on a study of the effects of commonly used drugs for heart disease (digitalis, anti-anginal, anti-thrombotic, and anti-arrhythmic and anti-hypertensive drugs) on LCAT activity.

Furthermore as stated previously we are comparing our results obtained with our technique with that obtainable by GLC.

Dr. Boden, with a grant from you will be carrying out a series of experiments on dogs to determine the effects of nicotine on pancreatic and gastric function with particular reference to secretin. He has agreed to permit us to use blood from his dogs to determine the effects of nicotine on LCAT activity and on blood lipoproteins and hopefully to determine the origin and degregation of LCAT by sampling blood across the liver from the portal vein from the systemic vein and from a systemic artery. LCAT activity will be measured both by our technique and by GLC. It is anticipated that a solution to the problem should be definitively obtained once we have developed a radio-immune assay technique.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

SPACE: The facilities available for this work include a fully equipped biochemical laboratory with space for at least four investigators and an office with two desks. A small separate room is presently being made available to house our gas liquid chromatograph and digital integrator accessory. A cold-room, shared by three other investigators is located in the "old Medical School" building which has recently been extensively renovated and re-equipped for research.

EQUIPMENT: Hewlett Packard 5711 A Gas Chromatograph
 Hewlett Packard 7671 A Automatic Sampler for G.C.
 Perkins Elmer Automatic Amino-Acid Analyzer (Fels Institute)
 Beckman Model E Analytical Ultracentrifuge (Fels Institute)
 Beckman L3-50 Preparative Ultracentrifuge (Infectious Disease Section)
 Beckman Model L Preparative Ultracentrifuge
 Beckman LS-100 Scintillation Counter
 Nuclear Chicago Mark II Scintillation Counter
 Gilford 240 U.V. and Visible Spectrophotometer
 Beckman DU-2
 LKB Ultrarac Fraction Collectors
 Two Buchler Fractomettes 200 Fraction Collectors
 LKB Uvicord II Monitor with Recorder
 Radiometer pH Meter 51
 Radiometer pH Stat with TTT III Titrator
 Photovolt Densicord Gel Scanner with Integrator
 Radiometer Conductivity Meter
 Chromato Vue Ultraviolet Viewing Box
 Buchi Evaporator
 Techni-Lab Heat Block
 Mettler Automatic Semi-Micro Balance
 Mettler P1000 Automatic "rough" Balance
 Buchler Evapo-Mix
 Canalco Disc Gel Electrophoresis Apparatus
 Beckman Microzone Electrophoresis with Duostat Power Supply
 Eberbach Shaking Water Bath
 Miscellaneous Equipment for Column Chromatography and Thin Layer

Chromatography including three Peristaltic and one Piston Action Pump

I have been asked to supervise a Research Lipid Laboratory in the Department of Obstetrics and Gynecology which opened on January 14, 1974. In addition a Pediatric Lipid Clinic has been started at St. Christophers Hospital which is Temple's Pediatric Hospital. This will give us an opportunity of studying children at all ages. This new laboratory is well equipped and has adequate space for a large office and two large laboratories. This laboratory will give me control of any lipid studies we wish to do in the newborn and his relatives.

11. Additional Facilities required:

Densitometer with integrator for thin layer chromatograms (Schoeffel)
 This instrument will permit precise quantification and also save considerable technician's time.

12. Biographical sketches of investigator(s) and other professional personnel (append):

See appendix's 1-6

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See appendix 1, page 2

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14. First year budget:

A. Salaries (give names or state "to be recruited")

| Professional (give % time of investigator(s) even if no salary requested) | % time | Amount |
|---|-----------|--------|
| Soloff, Louis A. | 30% | ----- |
| Rutenberg, Harold L. | 20% | ----- |
| Lacko, Andras G. | 60% | 9,759 |
| Raja, K.G. | 100% | 11,660 |
| Nowotny, Alois H. Consultant | as needed | ----- |
| David, Joshua S.K. | 100% | ----- |

Technical

| | | |
|-------------|------|--------|
| Baldwin, F. | 100% | 11,762 |
| Nickols, M. | 100% | 9,400 |
| Jetter, F. | 25% | 2,800 |

Fringe Benefits @ 13%

5,900

Sub-Total for A 51,281

B. Consumable supplies (by major categories)

| | |
|--|-------|
| Chemicals, Radiochemicals, glassware, vials, caps, service, plasma, volunteers, etc. | 5,000 |
|--|-------|

Sub-Total for B 5,000

C. Other expenses (itemize)

| | |
|----------------------|-----|
| Travel | 500 |
| Secretarial Services | 500 |

Sub-Total for C 1,000

Running Total of A + B + C 57,281

D. Permanent equipment (itemize)

| | |
|---|--------|
| Densitometer with integrator for thin layer chromatograms (Schoeffel) | 10,000 |
|---|--------|

Sub-Total for D 10,000

E. Indirect costs (15% of A+B+C)

E 8,592

Total request \$ 75,873

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | 53,845 | 5,250 | 1,050 | ----- | 9,022 | \$69,167 |
| Year 3 | 56,537 | 5,513 | 1,100 | ----- | 9,473 | \$72,623 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|------------------------------------|--------|--------------------|
| Physiological Studies on LCAT | Council for Tobacco Res. U.S.A. | 62,500 | 1973-74 |
| Purification and Chemical Characterization of Human Plasma LCAT | NIH | 92,673 | 1973-76 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| | | | |
| | | | |
| | | | |
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| | | | |
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University Health Sciences Center
% Mr. David W. Siegel

Mailing address for checks

3400 North Broad Street
Phila. Penna. 19140

Principal investigator

Typed Name Louis A. Soloff, M.D.

Signature *Louis A. Soloff* Date 1/21/74

Telephone 215 221 - 3343
Area Code Number Extension

Responsible officer of institution

Typed Name Samuel S. Herman, Ph.D.

Title Associate Vice President for Research

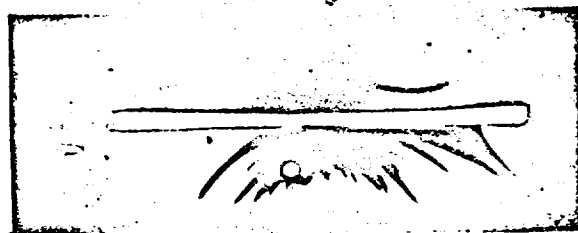
Signature *Samuel S. Herman*

Telephone 215 221-3250
Area Code Number Extension

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Fig. 1

519C



Preliminary additional work suggests that this arc is the enzyme and not enzyme contaminated with HDL or albumin.

1003540291

WATKINS

1003540292

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1974

Grant application #974

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson and Wyatt

Subject: Stephen Vatner, M.D., Peter Bent Brigham Hospital
New application No. 974
"Nicotine induced reflex coronary vasodilation"

History

This proposal was initially handled as case No. 210. Dr. Bing kindly visited Dr. Vatner in October 1973; enclosed is a copy of his comments.

Request

Application No. 974 requests \$31,595 plus two additional years.

Document Submitted

Enclosed is application dated 1/28/74.

FWN:gh

Enclosures

F.W.N.
F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH--U.S.A., INC.

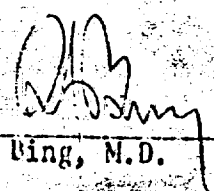
Application of Stephen Vatner

The application by Stephen Vatner deals primarily with establishment of a reflex arc, responsible for the action of the effect of nicotine on the coronary circulation. They have demonstrated this primarily by finding coronary vasodilation in the dog on deep inspiration. This coronary vasodilation occurs without an increase in systemic blood pressure and the afferent and efferent arches of this reflex system can be paralyzed with alpha and beta receptors respectively.

This is a rather sophisticated work and the methods are extremely difficult. For example, all experiments are carried out on completely unanesthetized dogs with implanted Doppler velocity measurements around the coronary arteries and measurements of ventricular volumes by means of sonar. Everything is recorded on tapes.

Unquestionably these workers are very knowledgeable and the whole business of a coronary reflex as induced by nicotine is a fascinating one. I am not so convinced as yet that hemodynamic parameters do not play a role, but even if this should be the case, it represents a most interesting project.

I therefore would recommend that Drs. Vatner and Braunwald be encouraged to submit a regular application to the CTR to be acted upon in March.


R. J. Bing, M.D.

RJB:cc

1003540294

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 4 1974

Date: 1/28/74

1. Principal Investigator (give title and degrees):

Stephen F. Vatner, B.A., M.D.

Assistant Professor of Medicine, Harvard Medical School and Associate in Medicine, Peter Bent Brigham Hospital.

2. Institution & address:

Peter Bent Brigham Hospital
721 Huntington Avenue
Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:

Department of Medicine, Peter Bent Brigham Hospital

4. Short title of study:

Nicotine induced reflex coronary vasodilation.

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years.

7. Brief description of specific research aims:

- 1.) The effects of intravenous nicotine, 4-20 $\mu\text{g}/\text{kg}$, on the integrated control of heart rate, arterial pressure, myocardial contractility, cardiac output, total peripheral resistance and blood flow and resistance in the coronary, mesenteric, renal and iliac beds will be determined.
- 2.) The extent to which intravenous nicotine increases coronary blood flow and reduces coronary vascular resistance will be determined. The extent to which nicotine induced coronary vasodilatation is primary or secondary to changes in myocardial O_2 requirements will be examined by comparing effects of intravenous and intracarotid nicotine on coronary dynamics: a) when the heart is in spontaneous rhythm, b) with heart rate controlled by pacing and c) after inotropic effects are blocked with propranolol, 1 mg/kg. In addition, arterial and coronary sinus oxygen will be measured to determine if coronary A- VO_2 difference narrows.
- 3.) The efferent autonomic pathways involved in the changes in heart rate, total peripheral resistance and resistances in the coronary, mesenteric, renal and iliac beds will be determined by selective and combined blockades of beta adrenergic receptors with propranolol, 1 mg/kg, alpha adrenergic receptors with phentolamine, 1 mg/kg and cholinergic receptors with atropine, 0.2 mg/kg.

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- 4.) The extent to which nicotine induced circulatory changes, and the induced coronary dilatation in particular, are due to nicotine's chemoreceptor stimulating action will be determined by injecting 0.2-0.4 $\mu\text{g/kg}$ nicotine through catheters previously implanted in the common carotid artery just proximal to the carotid sinus.
- 5.) The extent to which hyperventilation, through stimulation of pulmonary stretch receptors, resulting from nicotine's stimulation of chemoreceptors, regulates the circulation and specifically causes coronary dilation will also be examined by hyperinflating the lungs in conscious animals. The effects of pulmonary hyperinflation on heart rate, arterial pressure, myocardial contractility, cardiac output, total peripheral resistance and blood flow and resistance in the coronary, mesenteric, renal and iliac beds will be examined.
- 6.) The efferent pathways involved in the reflex pathways described in specific aims #4 and #5 will be examined as outlined in specific aim #3 above.
- 7.) Whether cigarette smoke can elicit coronary vasodilation in the conscious animal will also be determined by examining the responses of coronary blood flow, arterial pressure and left ventricular function to cigarette smoke inhaled through a tracheostomy.
- 8.) The above responses, specific aims #1-5, will be compared in the same animals after general anesthesia to demonstrate the importance of conducting this line of research in the conscious animal and to determine how general anesthesia modifies this aspect of reflex control of the circulation.

1003540296

Preliminary work in our laboratory indicates that intravenous nicotine produces striking coronary vasodilatation in the conscious animal (Fig. 1). The goal of this study is to delineate the mechanism by which this coronary vasodilation occurs. Specifically, it is proposed to 1) demonstrate that the nicotine induced coronary vasodilation is reflex in nature and 2) establish the afferent and efferent pathways involved in this response. It is known that nicotine stimulates chemoreceptors (1, 2), which in turn elicit hyperventilation (1, 2). It is proposed that the resultant hyperventilation, through stimulation of the lung inflation reflex (3-8), is responsible for the coronary vasodilatation. Finally, the extent to which this aspect of reflex control of the coronary circulation stimulated by nicotine is applicable to the rest of the circulation, i.e., left ventricular function, cardiac output, regional blood flows and regional vascular resistances will be examined.

9. Details of experimental design and procedures (append extra pages as necessary)

BACKGROUND AND RATIONALE

Existing knowledge regarding the cardiovascular effects of nicotine is based primarily on experiments performed in isolated organs or in open chest, anesthetized animals (9, 10). However, general anesthesia affects the control of the circulation exerted by the central nervous system to a variable extent and alters every aspect of circulatory function including heart rate, the level of myocardial contractile state (11, 12), vascular resistance (13) and in particular, the coronary vascular bed (14) and the distribution of regional blood flows (12, 15). Therefore, responses to normal physiological stimuli and to commonly used pharmacological agents may be quite different in normal healthy animals than in anesthetized animals or isolated organs. The applicants have demonstrated in conscious animals important differences from traditionally held concepts derived from studies in anesthetized animals, in diverse areas such as baroreceptor control of the circulation (16, 17), the influence of increasing cardiac frequency on myocardial contractility (Bowditch phenomenon) (18), increasing afterload on cardiac function (Anrep effect) (19), hemorrhage (20), the effects of catecholamines (21-23) and cardiac glycosides (11, 24, 25).

It is of particular importance to study the effects of nicotine on the coronary circulation in conscious animals, since nicotine exerts a variety of effects on the cardiovascular system (9, 10). It has direct effects on peripheral vessels, liberates catecholamines from adrenergic receptors and the adrenal medulla, stimulates the chemoreceptors, autonomic ganglia and stimulates myocardial contractility and myocardial metabolism. Despite the numerous investigations on the cardiovascular effects of nicotine (9, 10), of chemoreceptor stimulation (1, 2) and of the pulmonary inflation reflex (3-8), the finding that nicotine through stimulation of chemoreceptor and secondarily, the pulmonary inflation reflex, causes striking coronary vasodilation, has not been reported. The goal of this study is to elucidate these effects of nicotine in the conscious animal model.

METHODS OF PROCEDURE

1. Implantation of Transducers. Two general preparations will be employed. One series of mongrel dogs, 20-35 kg, will be instrumented for studies involving control of cardiac output, total peripheral resistance and the distribution of regional blood flows and resistances. Another series of dogs will be instrumented for studies involving control of the coronary circulation, left ventricle and myocardial contractility. All operations will be conducted using sterile

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surgical technique and pentobarbital, Na 30 mg/kg for anesthesia. The first series of dogs will be instrumented through a left thoracotomy, with Doppler ultrasonic or electromagnetic aortic flow probes on the ascending aorta for cardiac output, miniature pressure gauges in the aorta for arterial pressure and through a midline laparotomy with Doppler or electromagnetic pressure gauges on the mesenteric, renal and external iliac arteries. The second series of dogs will be instrumented through a left thoracotomy with Doppler or electromagnetic flow transducers on the left circumflex artery, miniature pressure gauges in the left ventricle for pressure, ultrasonic dimension transducers on opposing endocardial surfaces of the left ventricle for diameter and stimulating electrodes on the left atrium to maintain heart rate constant, and a Tygon catheter in the aorta to sample blood pressure and for arterial blood gas determination. In several of these dogs at a subsequent operation, using a right thoracotomy approach, catheters will be implanted chronically in the coronary sinus for coronary venous samples and coronary A-VO₂ determinations and in the right atrium for right atrial pressure.

For the series of experiments involving the role of pulmonary inflation reflexes stimulated indirectly via nicotine induced chemoreflex stimulation, PE-90 catheters will be implanted in the common carotid arteries proximal to the carotid sinuses.

2. Instrumentation Technique.

A. Measurement of Blood Flow. The ultrasonic Doppler flowmeter as developed by Franklin (26, 27) utilizes the principle that ultrasound reflected from a moving target, e.g., blood cells, exhibits a shift in frequency proportional to the velocity of the target. Continuous sonic energy is directed diagonally into the blood stream and a part of this energy is reflected from moving blood cells. The shift in frequency caused by this process provides an average of the instantaneous velocity of the red cells and is determined by extracting the frequency difference between transmitted and reflected sonic waves. An attractive and important feature of this flow system is the accurate electrical zero reference which does not require the additional implantation of an occluder to determine zero blood flow. When blood flow ceases, there is no Doppler shift; accordingly, the received ultrasonic signal is identical to the transmitted signal indicating zero flow.

A measure of volume flow is extracted by knowledge of the average velocity and internal cross-sectional area of the vessel. The relationship between velocity as measured by the Doppler flowmeter, and volume flow is linear as long as the cross-sectional area of the blood vessel within the transducer remains constant. This linear relationship between velocity and volume flow has been demonstrated repeatedly previously and confirmed by means of timed collections of blood flow (27). At autopsy, it has been observed consistently that the vessel is firmly adherent to the flow transducers through a fibrous scar which mitoses changes in the cross-sectional area of the blood vessel within the flow transducer. Additionally, the scale factor for the flow measurement remains stable under the connective tissue growth has had time to fix the transducer to the blood vessel.

An electromagnetic flowmeter is more appropriate for certain experiments. For instance, measurement of ascending aortic blood flow and in situations of significant reverse flow. In these experiments a square wave electromagnetic flowmeter (Statham SP2200) will be employed.

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B. System for Measurement of Arterial and Left Ventricular Pressures. Arterial pressure will be measured by means of a miniature intravascular pressure gauge as developed by Van Citters and Franklin (28), which will be implanted into the thoracic or abdominal aorta through a 1-2 cm. longitudinal incision closed by interrupted sutures to measure arterial pressure or implanted through a stab wound in the apex of the left ventricle and closed by a purse string suture to measure left ventricular pressure (28). The pressure gauge¹ consists of a titanium diaphragm, 5 mm. in diameter, which has bonded on its inner surface a silicon element Wheatstone bridge, which provides high fidelity signals proportional to blood pressure. This unit is sealed and thus measures absolute pressure. The signal output of the pressure gauge is 30 mV for 300 mmHg pressure change when excited by 5 volts DC.

C. System for Measurement of Left Ventricular Diameter. A measurement of left ventricular diameter will be obtained by determining the acoustic transit time of 3 MHz ultrasound propagated between two piezoelectric crystals placed in opposition on the endocardial surfaces of the left ventricle. In operation, the dimension gauge as developed by Patrick and coworkers (29), provides electrical impulses to one of the crystals and senses the propagated ultrasound impulses received by the opposing crystal. Rectangular voltage pulses are generated, the duration of which are equivalent to the transit time of the acoustic impulses, and are time averaged to produce a voltage proportional to ventricular diameter.

3. Experimental Protocol

The experiments will be conducted with the animals in the conscious state. Two to four weeks after recovery from operation, when the animals are vigorous and healthy, and after the animals are trained and accustomed to the laboratory and its personnel.

A. Effects of Intravenous Nicotine. After control recordings at rest and with the dogs in the basal state, small doses of nicotine (40 ug/kg) will be injected intravenously as a bolus. The resultant effects of chemoreflex stimulation and accompanying hyperventilation will be examined on heart rate, arterial and left ventricular pressures, left ventricular dimensions, myocardial contractility, coronary and regional blood flows and resistances. The efferent mechanism will be analyzed by repeating this procedure after selective and combined blockades of beta adrenergic receptors with propranolol, 1 mg/kg, alpha adrenergic receptors with phentolamine, 1 mg/kg, and cholinergic blockade with atropine, 0.2 mg/kg. Studies on the coronary circulation and myocardial contractility will be conducted with heart rate maintained constant as well.

In order to separate chemoreflex and pulmonary inflation inputs, the experiments will be repeated on a separate day in the same animals during an infusion of succinylcholine, 2-4 mg/min, and with respiration controlled with a Harvard ventilator. Thus, the direct chemoreflex effects will remain intact and the indirect stimulation of pulmonary inflation reflexes will be eliminated. It is recognized that succinylcholine is not an anesthetic agent; accordingly, no procedures will be conducted that would not be well tolerated by the dogs in the conscious state with succinylcholine. Intubation will be performed after spraying the larynx with a topical anesthetic. This procedure has already been done in conscious man (30).

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B. Effects of Intracarotid Nicotine. In order to separate systemic effects of nicotine from its stimulation of chemoreceptors, nicotine will be injected in a bolus into the implanted carotid arterial catheters in small doses (0.2-0.4 ug/kg). The effects of intracarotid nicotine will be examined on the same parameters as mentioned in section A. above; 1) in spontaneous rhythm, 2) with heart rate constant, 3) after selective and combined autonomic blockades and 4) after succinylcholine infusion and with ventilation held constant.

C. Direct Stimulation of Pulmonary Inflation Reflexes. The same animals will be studied on a separate day during succinylcholine infusion. After steady state control measurements are recorded, ventilation will be rapidly changed, both in frequency and volume. The effects of brief periods of pulmonary hyperinflation on heart rate, arterial and left ventricular pressures, left ventricular function and myocardial contractility and blood flows and resistances in the coronary, mesenteric, renal and iliac beds will be examined. In the experiments involving the coronary circulation it will be important to measure right atrial pressure as well. The efferent autonomic pathways of these responses will be studied by repeating the procedure after selective and combined autonomic blockades.

D. Effects of Anesthesia in Modifying the Effects of Nicotine on the Circulation. The experiments described above in subsections A and B will be repeated after general anesthesia with pentobarbital, Na 30 mg/kg. These experiments will demonstrate the importance of conducting research that involves an interaction between the respiratory and cardiovascular systems in conscious animals and will demonstrate the effect of general anesthesia in modifying this aspect of ventilatory reflex control of the circulation.

E. Effects of Cigarette Smoke. The effects of cigarette smoke administered through a tracheostomy will be examined on the circulatory measurements described in section "A" above, to determine if the same effects, particularly, coronary dilatation, can be elicited with inhaled cigarette smoke as well as intravenous or intracarotid nicotine.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

1600 sq. ft. of laboratory space is currently being utilized by the principal investigator and his colleagues for studies of circulatory function in conscious animals. This laboratory, as well as existing equipment including tape recorder, 8 channel oscillograph, and instruments for the measurement and radio telemetry of blood flow, pressure and dimensions will be available for this research. These projects will also utilize existing animal facilities at Harvard Medical School, Children's Hospital Research Building, and the New England Regional Primate Center.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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| | | | | | | |
|---|-------------------|----------------|------------------|----------------|-------|--------|
| 14. First year budget: | | | | | | |
| A. Salaries (give names or state "to be recruited") | | | | | | |
| Professional (give % time of investigator(s) even if no salary requested) | % time | Amount | | | | |
| S.F. Vatner, M.D. | 25% | 0 | | | | |
| E. Braunwald, M.D. | 5% | 0 | | | | |
| Technical | | | | | | |
| T. Manders - Research Assistant | 50% | \$5,000 | | | | |
| R. Peters - Electronics Technician | 50% | 5,000 | | | | |
| Fringe Benefits, 13.65% | | 1,365 | | | | |
| Sub-Total for A | | \$11,365 | | | | |
| B. Consumable supplies (by major categories) | | | | | | |
| 1. Dogs - 50 at \$50 | | 2,500 | | | | |
| 2. Boarding for dogs | | 1,500 | | | | |
| 3. Surgical Supplies | | 1,500 | | | | |
| 4. Miniature pressure gauge - 5 at \$500 each | | 2,500 | | | | |
| 5. Flow probes | | 2,000 | | | | |
| 6. Lab supplies | | 2,000 | | | | |
| Sub-Total for B | | 12,000 | | | | |
| C. Other expenses (itemize) | | | | | | |
| 1. Travel | | 500 | | | | |
| 2. Art and photography | | 250 | | | | |
| 3. Publication costs | | 250 | | | | |
| 4. Equipment maintenance | | 500 | | | | |
| Sub-Total for C | | 1,500 | | | | |
| Running Total of A + B + C | | 24,865 | | | | |
| D. Permanent equipment (itemize) | | | | | | |
| 1. Arterial blood gas analyzer | | 3,000 | | | | |
| Sub-Total for D | | 3,000 | | | | |
| E. Indirect costs (15% of A+B+C) | | 3,730 | | | | |
| Estimated future requirements: | | Total request | | | | |
| | | 31,595 | | | | |
| Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total | |
| Year 2 | 12,502 | 13,500 | 2,000 | 0 | 4,200 | 32,202 |
| Year 3 | 13,752 | 15,000 | 2,000 | 0 | 4,613 | 35,365 |

1003540302

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|---|----------------------|--------------------------|
| 1) Cardiovascular control in normal and disease states. E. Braunwald, M.D., Principal Investigator. | 1 R01 HL15416-02 - N.I.H. | \$92,240 | 1/1/73 - 12/31/77 |
| 2) Cardiovascular regulation in conscious animals. | Established Investigator - American Heart Association | - | July, 1974 - June, 1979 |
| 3) Effects of nicotine and cigarette smoke on coronary dynamics, blood flow distribution and myocardial contractility in conscious dogs. S.F. Vatner, M.D. Principal Investigator. | American Medical Association Grant # 407 | \$19,985 | Nov.1,1972 - Oct.31,1974 |
| 4) Cardiovascular Control mechanism in conscious baboons. S.F. Vatner, M.D., Principal Investigator. | American Heart Association | \$16,000 1st year | 7/1/73 - 6/30/76 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|-----------------|
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Stephen F. Vatner, M.D.

Signature Stephen P. Voth Date 11/31/74

Telephone 617 734-6000 3654
Area Code Number Extension

Checks payable to

Peter Bent Brigham Hospital

Mailing address for checks

721 Huntington Avenue

Boston, Massachusetts 02115

Responsible officer of institution

Typed Name William E. Hassan, Jr., Ph.D.

Title: Director, Peter Bent Brigham Hospital

Signature William E. Hansen Date 7/31/79

Telephone 617 734-8000 2101
Area Code Number Extension

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NICOTINE 4 μ g INTRACAROTID

L. CIRCUMFLEX
CORONARY
FLOW
(ml/min)

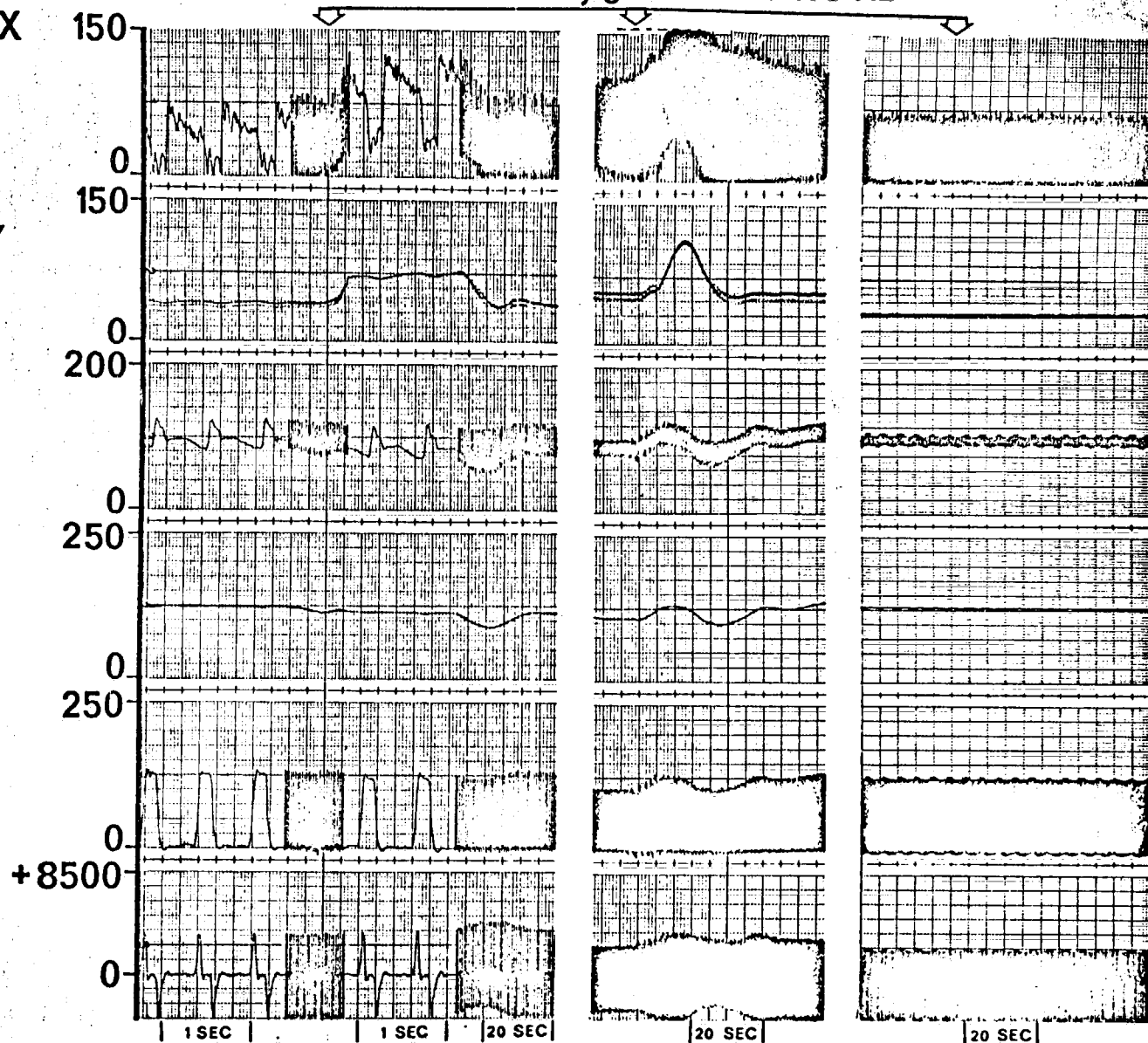
MEAN
CORONARY
FLOW
(ml/min)

ARTERIAL
PRESSURE
(mmHg)

MEAN
PRESSURE
(mmHg)

LV
PRESSURE
(mmHg)

dP/dt
(mmHg/sec)



CONSCIOUS SPONTANEOUS
NO BLOCK

VENTILATION
 β -CHOLINERGIC
BLOCK

ANESTHETIZED
CONTROLLED
VENTILATION

1003540304

Figure 1: Responses to intracarotid nicotine, 4 ug, in the same dog, conscious and with spontaneous ventilation (left panel), after beta receptor and cholinergic blockades (middle panel), and with controlled ventilation after pentobarbital, Na anesthesia (right panel). Responses of phasic and mean left circumflex coronary flow, phasic and mean arterial pressure, left ventricular pressure and dp/dt are shown. Note the striking increase in coronary flow associated with chemoreceptor stimulation and hyperventilation in the conscious animal (left and middle panels) and the absence of virtually any response at this dose of nicotine in the same animal after anesthesia (right panel).

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PHARMACOLOGY

1003540306

#6236 ESSMANN

1003540302

November 29, 1973

Grant application No. 623C

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner, Sommers

Subject: Walter B. Essman, M.D., Ph.D., Queens College of the City
University of N. Y.
Continuation application No. 623C
"Studies of Nicotine Action Upon Memory Consolidation"

History

Dr. Essman's grant for this study (one of two he has from CTR) has been active since the beginning of 1968. His current support, in the amount of \$21,579, was awarded as "terminal". Hence his enclosed request is in our parlance a "continuation", meaning that it competes as a new grant.

Request

Application No. 623C requests \$22,567 plus two additional years.

Documents Submitted

1. Attached is progress report, January 16, 1973 - November 15, 1973 (14 pages).
2. Also attached is application dated 11/15/73.
3. Reprints of the five publications listed on page 3b were provided. They constitute a stack an inch and a half high (Dr. Essman is never at a loss for words) and will be forwarded only as you request.

Also submitted was another inch and a half stack of earlier publications most of which you have seen before.

Comment

Dr. Essman's medical degree, a source of some puzzlement over the last few years, is now explained on page 3a of the enclosed application.

FWN:wg

JMN
F.W.N.

1003540308

Comm.

Dr. Bing
Dr. Gardner
Dr. Sommers

PHARMACOLOGY

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8343

Application for Research Grant
(Use extra pages as needed)

NOV 26 1973

Date: 11/15/73

1. Principal Investigator (give title and degrees):
Walter B. Essman, M.D., Ph.D.
Professor of Psychology & Biochemistry
2. Institution & address: Queens College of the City University of N.Y. and The Research Foundation of the City University of N.Y.
65-30 Kissena Blvd. 1411 Broadway
Flushing, New York 11367 New York, New York 10018
3. Department(s) where research will be done or collaboration provided:
Psychology, Biochemistry
4. Short title of study: "Studies of Nicotine Action Upon Memory Consolidation"
5. Proposed starting date: April 1, 1974
6. Estimated time to complete: 36 months (3 years)
7. Brief description of specific research aims:

The aims of the proposed research are to extend the current active work concerned with the relationship between nicotine action upon regional cellular and subcellular sites in the central nervous system to the process of memory consolidation. Such investigation will, more specifically, concern (1) the effects of nicotine action as a function of development; (2) the effect of nicotine and several of its metabolites in several inbred strains of mice, utilizing the proposed methods in this investigation; (3) the relationship of age as well as strain to centrally induced amnesia phenomena and their interaction with nicotine and (4) concern the relationship between nicotine effect and memory consolidation on a cellular level.

The aims thereby, of this proposed project are to relate two important parameters to the findings that have already emerged in the relationship between nicotine action,

continued on next page---

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7 Brief description of specific research aims: continued from previous page.

metabolism, and mediation of central aminergic changes to memory consolidation. Because of the potential significant contribution that nicotine holds for extending the memory consolidation process, and on more pragmatic grounds, for nicotine neuropharmacology, the important parameters of age and strain would appear to warrant more detailed consideration in this context.

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Within defined parameters of time and dosage, nicotine and several of its centrally active metabolites can alter the time course and nature of the memory consolidation process; also, the relationship between this observation and several cellular and regional alterations in amine metabolism might be modified; the facilitation of memory consolidation by nicotine treatment can be modified as a function of age from the specific strain in which treatment regimen is being observed. Endogenous differences in the regional and cellular characteristics of these biogenic amines with which both nicotine or amnesic agents or effects interact, is determined by age differences and/or strain differences that will contribute to a more fundamental understanding of the relationship between nicotine action and memory consolidation.

9. Details of experimental design and procedures (append extra pages as necessary)

The behavioral portion of all the experiments to be carried out will employ the single trial conditioning procedure, previously described in our protocols for establishing a stable avoidance response in one trial. This response, which is stable over time as well as within experimental conditions, has been successfully utilized to assess the amnesia effect of electroconvulsive shock, and other agents or events which, when presented in close temporal proximity with the acquisition trial experience, result in a reduction in the incidence of retention, as measured 24 hours later; amnesia and/or retention are assessed by measures of response latency so that criterion avoidance is defined on the basis of latencies in excess of five standard deviations of the mean latency exhibited on the training trial and an absence of retention, or retrograde amnesia, is defined as a response latency on the testing trial that is equivalent to or within one standard deviation of the mean response latency shown on the training trial. This procedure has been successfully utilized in our laboratory with over 20,000 mice during the nine to ten years within which the technique was originally devised by this investigation. A more detailed description of procedure, parameters governing acquisition and retention, and some of the applications thereof, may be found in several publications (Essman & Alpern, 1965; Essman, 1968; Essman and man, 1969).

Animals utilized in all of the proposed studies will be mice that will either be

9. Details of experimental design and procedures -- continued from previous page.

obtained from a commercial vendor or specifically bred through vivarium facilities located within the investigator's laboratory, for precise control over age differences. The strains of mice to be utilized will include CF-1S strain (Carworth Farms, New City, New York), which have been employed throughout the course of previous investigation; C-57 BL/6J, a strain of mouse which in previous experiments has shown highly efficient learning ability, has demonstrated a profoundly decreased susceptibility to the amnesic effects of several post-training treatments producing retrograde amnesia. Previous experience with this strain has included only learning ability animals, and the use of this strain with nicotine has not been employed. It is also our experience that whole-brain 5-HT levels in this strain are somewhat higher ($0.75 \mu\text{g/g}$) than average values ($0.49 \mu\text{g/g}$) obtained at comparable ages for CF-1S strain mice. A somewhat more emotionally labile mouse will be selected for the 3rd strain included within this series, the DBA strain. This strain of animal has, in previous experience, been shown to be an extremely poor candidate for most of the studies in which it has been employed. The animal is a poor learner under conditions where response acquisition has been demonstrable; increasing susceptibility to amnesic agents and/or events, has been shown. The purpose in using 3 different strains of the genus *Mus musculus*, is to provide for age differences as well as differences which, in the mature mouse, occur in relation to amnesia susceptibility.

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Animals from the designated strains will be utilized from 16 to 35 days of age and the specific experiments will concern: (1) effects of nicotine and its active metabolites upon memory consolidation during early development among several strains of the genus *Mus musculus*. Effects of two amnesic treatments will be independently assessed in this group of mice. Transcorneal ECS or intrahippocampal 5-HT, as have been previously documented in the CF-1S mouse, which in present studies will constitute a baseline, will be given following single trial passive avoidance conditioning

in nicotine treated mice. The temporal gradient for the expected retrograde amnesia effect of such treatments will be assessed as a function of age in which stress relationship emerges between drug treatment and the antagonism of retrograde amnesia will allow for the specification of the parameters within which subsequent experiments may be carried out; (2) Identification of the temporal relationship between the effect of nicotine and/or metabolites and cerebral amine metabolism. On the basis of previous findings, it seems appropriate to pursue the hypothesis that nicotine induced antagonism of a retrograde amnesia stimulus depends upon the ability of such treatment to block the neurochemical sequela of the amnesic stimulus. Such sequela have been specifically, brain biogenic amines, notably 5-hydroxytryptamine. Therefore, in relation to the time course of the aminergic effect of nicotine on several agents in several strains of mice, the interaction will be studied for both ECS and intra-hippocampal 5-HT as predictable amnesic-inducing agents. (3) Specific cellular effects of nicotine and these metabolites as related to memory consolidation in several strains of mouse during late development. The relationship between developmental age and the susceptibility of amnesic agents or events has emerged as dependent upon the status of brain amines, their lability in relation to amnesic stimuli, and the influence of such alterations upon cerebral protein synthesis at specific cellular sites. This series of experiments will be directed toward the elaboration of age and strain variables as they contribute to amine regulation of protein synthesis. This relationship will be explored in animals treated with nicotine and several of its active metabolites and the effects of such treatment upon 5-HT-and protein synthesis-changes produced by amnesic stimuli. These relationships will be examined on a regional, cellular and subcellular level and if sufficient time permits, attempts will be further made at the identification of the proteins, which for specific age populations or strains, becomes resistant and/or susceptible to inhibition by amnesic stimuli.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Two air-conditioned laboratories with adjoining vivarium facilities are currently in use by the investigator. All equipment necessary for the behavioral and biochemical procedures involved with this proposal are available and no additional equipment needs are anticipated for the duration of this project.

11. Additional facilities required:

NONE

1003540314

12. Biographical sketches of investigator(s) and other professional personnel (append): See Page 3a.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See Page 3b.

- 3a -

12. Biographical Sketch of Investigator:

WALTER B. ESSMAN

Education:

- Ph.D. - University of North Dakota, R- (Major: Psychology;
Minor: Medical Sciences)
- M.D. - (Cum Laude) - University of Milan, R
- M.A. - University of North Dakota, R
- B.A. - New York University, R

Experience:

Professor (Psychology, Biochemistry), Queens College of the City
University of New York, 1967 - Present.

Associate Professor, Queens College of the City University of New
York, 1965-66.

Assistant Professor, Queens College of the City University of New
York, 1962-64.

Research Professor, Neurology, Mt. Sinai School of Medicine, New
York, 1972 - Present.

Research Associate, Laboratory of Neurochemistry, Mt. Sinai School of
Medicine, New York, 1966-72.

Research Fellow, Laboratory of Neurochemistry, Mt. Sinai Hospital,
New York, 1964-66.

Research Assistant Professor, Dept. of Rehabilitative Medicine, Albert
Einstein College of Medicine, 1962-63.

Research Associate, Dept. of Physiology, Albert Einstein College of
Medicine, 1961-63.

Senior Post-Doctoral Fellow, Neurophysiology, Albert Einstein College
of Medicine, 1959-61.

Director, Psychophysiological Research, U.S. Army Surgical Research
Unit, 1958-59.

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13. Publications (5 most recent):

1. Essman, W.B. Neurochemistry of Cerebral Electroshock. New York: Spectrum Publ., 1973. ✓
2. Essman, W.B. Neuromolecular modulation of experimentally induced retrograde amnesia. Confinia Neurol., 1973, 35: 1-22. ✓
3. Essman, W.B. Effects of ECS on cerebral protein synthesis. In: Fink, M., Kety, S.S., McGaugh, J., & Williams, T. (Eds.). The Psychobiology of Convulsive Therapy. Washington, D.C.: V.H. Winston & Sons, 1974, 237-249. ✓
4. Essman, W.B. Drug effects and learning and memory processes. In: Garattini, S. and Shore, P. (Eds.). Advances in Pharmacology and Chemotherapy. New York: Academic Press, 1971, Pp. 241-330. ✓
5. Essman, W.B. Changes in cholinergic activity and avoidance behavior by nicotine in differentially housed mice. Int. J. Neurosci., 1971, 2: 199-206. ✓

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14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)

Dr. Walter B. Essman

100%

Technical

Traum, John - Technical Assistant
Rosenthal, Richard -- Assistant

100%

50%

+16% Fringe Benefits

Sub-Total for A

B. Consumable supplies (by major categories)

Animal Purchases

800.

" Cleaning, Bedding

600.

Chemicals, Glassware

840.

Disposable Cages

450.

Sub-Total for B

\$2,690.

C. Other expenses (itemize)

International Symposium on Memory

Processing, Milan, Italy, June '74

Endocrine Society, Chicago, Ill., June '74

American College of Neuropsychopharmacology,

San Juan, Puerto Rico, Dec. '74.

Sub-Total for C

\$1,100.

Running Total of A + B + C

\$19,624.

D. Permanent equipment: (itemize)

NONE

Sub-Total for D

E

\$ 2,943.60

Total request

\$22,567.60

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|-------------|
| Year 2 | R | \$2,690. | \$1,100. | --- | \$2,943.60 | \$22,567.60 |
| Year 3 | | \$2,690. | \$1,100. | --- | \$2,943.60 | \$22,567.60 |

REDACTED

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5.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

| CURRENTLY ACTIVE | | | |
|--|--------------------------------|-----------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| Metabolic Response to Stress -- Tobacco Smoke Interactions | RF-09358 C.T.R. #836A | \$44,000. | 10/1/73 - 9/30/74 |
| Role of Nicotine Action Upon Memory Consolidation | RF-09410 C.T.R. #623BR1 | \$21,579. | 4/1/73 - 3/31/74 |

| PENDING OR PLANNED | | | |
|--------------------|--------------------------------|--------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Principal investigator

Typed Name Walter B. Essman, M.D., Ph D.Signature [Signature] Date 11/15/73Telephone [Signature] R

Area Code Number Extension

Checks payable to

Research Foundation of C.U.N.Y.

Mailing address for checks

Mr. Paul Segall, ComptrollerResearch Foundation of C.U.N.Y.1411 Broadway
New York, N.Y. 10018

Responsible officer of institution

1. Hannah PetzenbaumTyped Name 2. David H. Speidel1. Research Foundation - Queens CollegeTitle 2. Associate Dean of the Faculty - Q.C.Signature [Signature] Date 11/16/73[Signature]

Area Code Number Extension

1003540318

#970 - MCKENNETT

1003540319

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

February 14, 1974

Grant application No. 970

PHARMACOLOGY

To: The committee comprising Drs. Bing, Jacobson, and Sommers

Subject: Herbert McKennis, Jr., Ph.D., Medical College of Virginia, Richmond
New application No. 970
"Pharmacodynamics of Cotinine"

History

CTR grants to Dr. McKennis, under various titles, began back in 1956.

In October 1973 a renewal request "Biological Activity of Tobacco Smoke Components and Allied Substances" was denied. Terminal support to April 1, 1974 was provided to help ". . . in making orderly staff and program readjustments . . .".

The enclosed proposal has a new title and appears to attempt somewhat modified emphasis.

Request

Application No. 970 requests \$38,662 plus two additional years. The budget is wholly for salaries of Drs. E. R. and F. J. Bowman, previously compensated from CTR grants.

Documents Submitted

Attached is application dated 1/17/74.

Comment

Dr. Hockett may wish to comment, particularly since he has had recent discussions with Dr. McKennis.

FWN:gh
Encls.


F.W.N.

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71671 1/21/74
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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/17/74

1. Principal Investigator (give title and degrees):

Herbert McKennis, Jr., Ph.D.
Professor of Pharmacology

2. Institution & address:

Department of Pharmacology
Medical College of Virginia
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology, Medical College of Virginia
Department of Chemistry, Duke University
Department of Toxicology, Karolinska Institutet, Stockholm, Sweden

4. Short title of study:

Pharmacodynamics of Cotinine

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: Three years (with publishable results within first year).

7. Brief description of specific research aims:

The specific research aims on the pharmacodynamics of cotinine, a principal metabolite of nicotine, are directed toward an understanding of the possible contribution and role of cotinine and its metabolites in physiological responses to nicotine. Since the list of metabolites is lengthy and real or alleged responses to smoking are numerous, it is the intent during the first year to emphasize only those aspects of the problem in which there are already well-established, interesting leads. These principle current leads arise primarily from past studies here and elsewhere on cardiovascular and lipid-metabolism effects and suggest that some of the metabolites of cotinine may serve to block through direct competition the action of nicotine at various receptor sites.

1003540321

8. Brief statement of working hypothesis:

The metabolism of nicotine via cotinine leads to a number of compounds in which there has been further alteration or degradation of the pyrrolidine ring of the parent nicotine. These transformations take place at varying rates depending upon genetic control, dietary and other factors which affect pH of physiological compartments and structures, rate of absorption, excretion, etc. This permits possible participation of numerous nicotine metabolites in various physiological functions and in the possible suppression of various effects attributed to nicotine by itself. The extent to which these effects may be observed will be dependent in part upon rates of formation and on degree of concentration of the metabolites in various parts of the body. Various lines of evidence suggest that cotinine, which is detectable in the body long after nicotine has disappeared, may participate in producing some subtle physiological consequences which may become important as a result of future study.

After the initial isolation of cotinine as a mammalian metabolite of nicotine (McKennis, Turnbull, and Bowman, J. Amer. Chem. Soc. 79, 1342 (1957)), it was established that rather large quantities of cotinine administered to man and other animals under varying conditions produced

(continued)

9. Details of experimental design and procedures (append extra pages as necessary)

(see page 4)

1003540322

8. Brief statement of working hypothesis:

Continued...

no apparent adverse reactions. For example, in one experiment (McKennis, Turnbull and Bowman, J. Biol. Chem. 238, 720 (1963)) a male subject received 10.8 g of cotinine in ^{orally} divided doses ~~of~~ a six day period for the purpose of obtaining nicotine metabolites that are derived from the intermediate cotinine. No adverse effects were reported, and in another study lesser amounts of cotinine (approximately 50-100 mg per day orally for several weeks) were received orally by 33 subjects. The clinical reports are of such a nature as to suggest that cotinine is well-tolerated.

Recent data from various laboratories show a rapid disappearance of nicotine from the blood of human subjects following smoking (see, for example, Langone, Gjoka, and Van Vunakis, Biochemistry, 12, 5025 (1973)). The rapid fall in nicotine levels in the blood is followed by a rise in the reported cotinine levels in the serum. Cotinine is then excreted in the urine as itself or in the form of a variety of mammalian metabolites which include many in which there has been further chemical alteration of the pyrrolidine ring of the parent nicotine. The list of compounds thus implicated in the nicotine metabolism, which is rather extensive and has been previously reported in the literature and in reports from us to the Council for Tobacco Research, includes 3-pyridylacetic acid, 4-3-pyridyl-4-oxobutyric acid, 4-3-pyridyl-3-methylaminobutyric acid, 4-3-pyridyl-4-hydroxybutyric acid, 4-3-pyridyl-3-butenic acid, 4-3-pyridylbutyric acid, N-3-pyridylacetyl glycine, 3-hydroxycotinine, 5-hydroxycotinine, and demethylcotinine.

In essence, one may say that cotinine, which is clearly detectable in the blood many days after the cessation of smoking, represents a slow release form (via metabolism) of the listed substances.

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9. Details of experimental design and procedures:

For illustration, provisional experimental designs and procedures are briefly summarized in three areas in which some of the interesting results or leads already have been obtained.

I. Effect of Cotinine Metabolites on Lipid Metabolism

Various experimental procedures that have been employed in man to study the effect of numerous agents in depressing or elevating serum free fatty acid (FFA) levels have been described. In particular those related to smoking have been reviewed in the Tobacco monographs of Larson *et al.* Other aspects are revealed in Metabolic Effects of Nicotinic Acid and Derivatives, edited by Guy and Carlson, Hans Huber Publishers (1971). Some of the difficulties of such studies include stress or excitation to the subjects as a result of the experimental procedures. The stress may often mask the inherent activity of compounds under physiological investigation.

Recent comparative studies on the effects of various 3-pyridyl-alkyl carboxylic acids on preventing epinephrine-induced FFA rise in dogs. It becomes desirable to parallel the techniques described in the two cited by Carlson *et al.* in initial experiments. It may be noted that there already exists data (Acta Pharm. Suecica 9, 405 (1972)) attributing to 4-3-pyridyl-butyric acid on a molar basis a greater potency in suppressing FFA rise than that of 3-pyridylacetic acid. Both of these acids are involved in the metabolism of nicotine. By analogy with other data, 4-3-pyridyl-3-butenic acid and 4-3-pyridyl-4-hydroxybutyric acid may have similar interesting action in this regard. These substances, N-3-pyridylacetyl-glycine, and other substances in the degradation of cotinine deserve experimental consideration, since cotinine may be considered to be a slow-release form of the various compounds. Additionally, since 4-3-pyridylbutyric acid is probably metabolized to 3-pyridylacetic acid, the butyric acid derivative and other nicotine metabolites can be considered slow release forms of 3-pyridylacetate.

II. Inhibition of Direct and of Indirect Effects of Nicotine on Smooth Muscle

Various model systems of varying degrees of simplicity have already been employed in this laboratory to search for possible inhibitory control of the effects of nicotine on smooth muscle systems. The procedures which are based on whole or in part in application of standard literature to various pyridyl compounds and nicotine metabolites (Kim, Borzelleca, Bowman and McKennis, *J. Pharm. Exp. Therap.* 161, 59 (1968); Konzett, Bost, Bowman, Bowman, and McKennis, *J. Pharm. Exp. Therap.* 178 122 (1971); McKennis, Chang, Bowman, and Wilson, *Fed. Proc.* (1974) submitted recently and previously supplied by copy to the Council).

(a) Aortic Strips - A possible suggestion of validity already established for the experimental design arises from studies (recently completed and unpublished) on contraction of rabbit aortic strips. After first establishing a standard response to nicotine and metanictine, it was shown that pretreatment with two selected nicotine metabolites 3-pyridylacetic acid and N-3-pyridylglycine provide a partial or total block, inhibition of the contraction produced by nicotine or metanictine. Precise mathematical relationships between the dosages required for stimulation and blockade with the various substances remain to be determined.

(b) Isolated Intestinal Segments - Practicality of the use of isolated intestinal segments for studies *in vitro* on possible antagonism between nicotine metabolites and nicotine itself has been previously demonstrated (Kim, Borzelleca, Bowman and McKennis, *J. Pharm. Exp. Therap.* 161, 59 (1968)). In this early study it was shown that cotinine methonium ion blocked the response to nicotine, but not acetylcholine, barium chloride, or histamine, in segments obtained from the rabbit.

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(c) Peripheral Vascular Resistance in the Perfused Forelimb of the Dog

This model has been employed (Konzett et al, J. Pharm. Exp. Therap., 178, 122 (1971) in this laboratory to study the histamine-like effects of betahistine during the course of clinical work by other investigators, who noted improved circulation in the brain and other organs (excluding the kidney) as a result of oral administration of the compound. The use of this preparation for studies on nicotine and its metabolites has already been reported to the Council for Tobacco Research - USA. With some substances in the nicotine series a "trimodal" type of distribution in biological response was noted. It has been suggested to us that a definable genetic basis is implied by the type of distribution. Irrespective of interpretation, this type of variation has limited the usefulness of the preparation in many studies. However, the simplicity of the surgical procedure to provide blood delivery at constant flow with changes in resistance (pressure) readily measured makes the preparation useful adjunct in experimental studies.

(d) Possible Mechanism of Physiological and Biochemical Antagonism to Some of the Actions of Nicotine

Preliminary studies on the effect of diamine oxidase (pig kidney origin) on various nicotine metabolites were reported in a report to the Council (October 1, 1973). The data suggest that dihydrometanicotine and metanicotine, two metabolites of nicotine, are rapidly oxidized by the enzyme preparation and that these substances can inhibit histamine oxidation. Such studies are of interest if one considers that contraction of capillary sphincters is under adrenergic control and that this contraction is opposed by histamine. In other words some instances of improved or unimpaired microcirculation following exposure to nicotine may be ascribable to a biochemical protection of local histamine which may be capable of opposing the actions of local or peripherally released adrenergic substances. Other interpretations, including inducement of platelet deaggregation, are of course possible considerations. Enzymatic studies of this type and others have, of course, a direct and indirect bearing on central nervous system effects of nicotine.

(e) Chemical Considerations

In the sequence of known compounds leading from nicotine through cotinine to 3-pyridylacetate and its glycine conjugate, which is more potent than pyridylacetate as a nicotine antagonist in preliminary studies in vitro, methods for synthesis have already been described in publications from this laboratory and are cited as references or additional references in section 13. The most recently reported and most convenient route to 5-hydroxycotinine is via dibromoticonine. The method for synthesis of the latter appears in an attached reprint (J. Chem. Soc., Perkins Transactions I, 2046-2049 (1973)).

(f) General Comments

On the basis of preliminary studies already conducted, it is reasonable to anticipate an increasing number of instances in which pharmacological antagonism to nicotine by its metabolites may be uncovered. Since better assay methods for the metabolites of nicotine and nicotine itself are continually being developed throughout the world, it will become increasingly easier to interpret the possible significance of these pharmacological events. Already it has been demonstrated from the experiments of Schmitterl w et al and of Waddell that there is considerable difference in the distribution of the radioactivity of nicotine-C¹⁴-methyl (after injection of the compound into various strains in mice. This difference may reflect a difference of concentration of nicotine or metabolites in various areas of the body; such features may suggest a pharmacological activity of some metabolites as "anti-nicotines" in excess of that activity now projected from smoking data and existing data on cotinine levels in plasma which follow smoking of tobacco.

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(6)

Although not part of this study, there are additional features which make the study interesting to us. These include the possibility that some seemingly desirable central effects of nicotine may be mediated in whole or in part by peripheral effects. As an example, it has been considered that some of the desirable psychological consequences of cotinine reported by human subjects could be ascribed to a mild muscular relaxant property.

1003540326

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Two laboratories (approximate total of 800 sq. ft.), well-equipped for chemical and pharmacological studies, are available for these studies. In addition, there are two instrument rooms which house spectrographic, chromatographic, and radioactive counting equipment. Animal quarters (shared with others) are available for small mammals and large animals (horses, etc.) are kept in rented areas or at a school animal farm.

List of some major items of permanent equipment available for this work:

Cary recording spectrophotometer, model 11-PM
 Grass polygraph, six channel, model 5
 Nuclear-Chicago liquid scintillation system, 720 series
 Beckman amino acid analyzer, model 120B
 Perkin-Elmer gas chromatograph, model 801
 Nuclear Chicago gas chromatography counting system
 Wilkens Aerograph Autoprep, model A-700
 Preiser Scientific integrator-printer
 Wilkens Aerograph 200 (2 each)
 Nuclear-Chicago Actigraph III paper radio chromatography system
 International preparative ultra centrifuge, model B-35
 Vacuum pumps (six of various types)
 Warburg Apparatus
 Hewlett Packard Model 5700A Gas Chromatograph with integrator
 Chemical balances (4 each)
 Zeiss photoelectric polarimeter
 Cahn electrobalance

(continued)

11. Additional facilities required:

If any, this would be determined by the outcome of the investigations.

12. Biographical sketches of investigator(s) and other professional personnel (append):

(see page 8)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003540327

10. Space and facilities available:

Continued...

Fraction collectors (2 each)

Miscellaneous glass metabolism cages, distillation equipment, chromatography equipment

Radiometer pH meter, O_2CO_2 determinator

Blood oxygenator (local design for organ perfusion)

Varian A-60 NMR apparatus

DuPont Model 830 Liquid Chromatography Apparatus

12. Biographical sketches of investigator(s) and other professional personnel:

NOTE: These are appended for principal investigator and co-workers, who are experienced in areas included in proposed study. Those not included in 14A (salaries for the first year budget) may be attracted on a limited voluntary basis and a more extensive basis if funding can be later accomplished through sources which have not presented themselves or been solicited. (No formal or informal request has been made to other possible sources.)

1003540328

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)Herbert McKennis, Jr., Ph.D.
(Professor)

% time

Amount

20%

\$ -----

Edward R. Bowman, Ph.D.
(Research Associate)

100%

\$22,846.00

Faye J. Bowman, Ph.D.
(Research Associate)

70%

\$10,773.00

Arthur W. Burke, Jr., M.D., Ph.D.
(Resident in Radiology)

Undetermined

Technical

Kendall L. Wilson, Jr., M.S.
(Lab Specialist)

Undetermined

Sub-Total for A \$33,619.00

B. Consumable supplies (by major categories)

(FUNDS FOR CATEGORIES B, C, AND D TO BE SOUGHT ELSEWHERE)

Sub-Total for B

C. Other expenses (itemize)

Sub-Total for C Running Total of A + B + C \$33,619.00

D. Permanent equipment (itemize)

Sub-Total for D E \$ 5,042.85Total request \$38,661.85

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|-------------|-------------------|----------------|------------------|----------------|-------------|
| Year 2 | \$35,299.95 | | | | \$5,295.00 | \$40,594.94 |
| Year 3 | \$37,053.41 | | | | \$5,558.02 | \$42,611.43 |

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(10)

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|-----------|--------------------|
| Factors Controlling the Development of Pharmacologically Active Derivatives of Nicotine | American Medical Assoc. | \$45,750. | 7/1/73 - 6/30/74 |
| Biological Activity of Tobacco Smoke Components and Allied Substances | Council for Tobacco Research - USA #868 | \$30,000. | 10/1/73 - 4/1/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|------------------|--------------------------------|--------|--------------------|
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mr. P. Lossing, Comptroller-Treasurer

Mailing address for checks
VCU/MCV

1200 East Broad Street, Richmond, Va 23298

Principal investigator

Typed Name Dr. Herbert McKennis, Jr.

Signature Herbert McKennis, Jr. Date 1/17/74

Telephone (804) 770-4406

Area Code Number Extension

Responsible officer of institution

Typed Name M. Pinson Neal, Jr., M. D.

Title Provost VCU/MCV

Signature M. Pinson Neal, Jr. Date _____

Telephone (804) 770-5150

Area Code Number Extension

1003540330

#919 - RANA EASTRY

1003540331

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

November 29, 1973

Grant application No. 949

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner, and Sommers

Subject: B. V. Rama Sastry, D.Sc., Ph.D., Vanderbilt Univ., Nashville
New application No. 949
"Influence of Nicotine on the Release of Acetylcholine in
the Human Placenta and its Implications on the Fetal Growth"

History

This proposal was case No. 186 and application was encouraged.

Request

Application No. 949 requests \$12,806.00 plus two additional years.

Documents Submitted (attached)

1. Application dated 11-15-73.
2. Appendix. Only items 1 and 4 listed on the contents page of this Appendix are being forwarded to you enclosed. The remaining items are voluminous and not pressingly relevant; nevertheless they will be forwarded promptly if you request.

FWN:gh
Enclosures

JWM
F.W.N.

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Committee:

Dr. Bing
Dr. Gardner
Dr. Sommers

PHARMACOLOGY

#949

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant

(Use extra pages as needed)

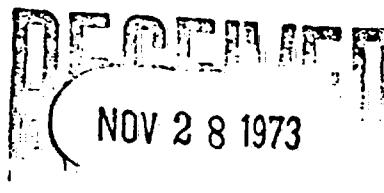
Date: 11-15-73

1. Principal Investigator (give title and degrees):

B.V. Rama Sastry, D.Sc., Ph.D.
Professor of Pharmacology

2. Institution & address:

Vanderbilt University
School of Medicine
Nashville, Tennessee 37232



3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Influence of Nicotine on the Release of Acetylcholine in the Human Placenta
and its Implications on the Fetal Growth

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The existence of an acetylcholine (ACh)-choline acetyltransferase (ChA)*-acetylcholinesterase (AChE)**-like system in the human placenta has been demonstrated by a number of workers. Since the placentae of man and animals lack innervation, a number of questions arise about (a) the similarities and differences between ACh-ChE-AChE systems of nervous and non-nervous sources, (b) the significance of the location of cholinergic system in the human placenta, (c) the degree of permeability of the placental barrier to drugs and environmental chemicals and (d) the influence of the cholinergic system on the nutritional health of the fetus and the maintenance of pregnancy.

The ChA's from placental and nervous sources have been investigated in our laboratories and they were found to be similar in their chemical properties and the enzyme mechanism (1-3). According to present indication, ChA is located in the chorionic villi, in the cell layers separating maternal and fetal blood compartments (4). The major component of the ACh-like substances in the human

* Recommended trivial name for Acetyl-CoA: choline O-acetyltransferase (2.3.1.6) by the International Union of Biochemistry. Other name: choline acetylase.

** Recommended trivial name for Acetylcholine hydrolase (3.1.1.7). Other names: true or specific cholinesterase.

1003540333

7. Specific Research Aims (cont'd.)

placenta has been identified as ACh by pyrolysis gas-chromatography (5). ACh was present in a bound form in the placenta. There is some experimental evidence that the bound ACh-like substances are located in "granules" which are liberated from the trophoblast epithelium (6).

In view of the high concentration and the location of ACh in the placenta, and the lack of any significant effect of ACh on the placental vascular system, (7-9) one is apt to suggest that the permeability of the placental barrier is controlled by the quantal release of ACh. If this statement were true, ACh-ChA-ChE system is of importance in the placental function during pregnancy. This placental function may be related to the transport of materials across the placental barrier, or release of placental hormones by ACh during pregnancy. A significant number of observations indicate the importance of the placental cholinergic system in the maintenance of pregnancy and the health of the fetus. Our results indicate that abortive human placenta contains higher levels of ACh than terminal placenta. Administration of anticholinesterase agents to pregnant mice causes the death of fetii (10). Ingestion of drugs with anticholinesterase activity as a side effect (e.g. morphine) induces fetal malformations in mice (11).

Cholinomimetics (e.g. pilocarpine) have been shown to exhibit anti-growth (or teratologic?) effects during chick morphogenesis (12). Some investigators have suggested that cholinomimetics produced the above conditions by affecting the hypothetical relationship between the cholinergic system and growth and morphogenesis (12) or by interfering with energy metabolism under *in vivo* conditions (13,14). However, there is no available evidence of the possible effect of cholinomimetics or quaternary ammonium compounds on the multi-step processes involved in the transport and metabolism of nutrients *in vivo*.

Recent surveys indicate that babies of women who are habituated to smoke tobacco are smaller in size than those of nonsmokers (15,16). Further recent studies indicate that nicotine releases ACh from presynaptic nerve terminals and synaptosomes (17,18). In view of these observations, one could anticipate that nicotine can release more than the normal quanta of ACh from its stores in the villous epithelium. Released ACh (and/or nicotine) may influence the transport of energy metabolites across the trophoblast. The final result would be the fetal deprivation and relatively poor fetal growth (Fig. 1). At the present time it is not possible to assess whether the final effect is due to released ACh or nicotine or both.

No studies are available on the effect of nicotine on ACh stores in placenta. Therefore, this application is made for evaluation of the following specific aims:

1. To study the effects of nicotine on the release of ACh from human placental villi and to establish relationships between the dose of nicotine and ACh released into the medium (or ACh retained in villi). The exact concentrations of ACh will be analyzed by pyrolysis gas chromatography.
2. To separate the ACh granules from placental tissue by density gradient separation, and to study the mechanisms of ACh release from granules by nicotine. The results in specific aim (1) lead to specific aim (2).

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7. Specific Research Aims (cont'd.)

3. To obtain evidence for the presence of a cholinergic receptor in the human trophoblast where nicotine binds for exhibiting agonistic or antagonistic effects. The results in specific aims (1) and (2) lead to specific aim (3).

4. To set up a perfusion system for human placenta for use in our laboratories. This system is useful for studying (a) the transport of drugs, energy metabolites or ions across the placental barrier during manipulation of placental cholinergic system by nicotine and other pharmacological agents, (b) the effects of nicotine on ACh release and transport of energy metabolites across placental barrier simultaneously, and (c) the effects of nicotine in human placenta collected at different gestation periods. Studies in specific aims (1) - (3) lead to studies in specific aim (4).

1003540335

8. Brief statement of working hypothesis:

The placenta plays an important role in the transport of materials between mother and fetus in a most complex way (Fig. 1). This role may be related to the active involvement of placenta in assisting and regulating transport of water soluble and ionizable materials. According to our present indications, ChA is located in the chorionic villi, in the cell layers separating maternal and fetal compartments. According to our investigations, ACh is located in chorionic villi in a bound form, possibly as granules whose contents may be released from the trophoblast epithelium. Free ACh may stimulate a cholinergic receptor on the trophoblast epithelium and thereby induce changes in its permeability to charged molecules. One would anticipate rhythmic fluctuations in the permeability of the trophoblast epithelium with constant shifting of the dominant site, which in turn may be dependent of the amount of ACh released. The changes in the permeability of the trophoblast epithelium facilitates the exposure of the various molecules on the maternal side to their corresponding carriers (or the transport processes). In other words, ACh stimulation of the cholinergic receptor in the trophoblast is coupled to the transport of substances across the placental barrier. This "ACh stimulation-transport coupling" is delicately balanced by the amount of ACh released. Both very low and high concentrations of free ACh are deleterious to the fetus.

According to the above hypothesis, nicotine may have two sites of action. Nicotine may mimic ACh and stimulate the cholinergic receptor in the trophoblast. Cholinomimetics are known for exhibiting antigrowth effects. Nicotine is also known for exhibiting antagonistic effects at high concentrations. Further, nicotine may release excessive quanta of ACh from its bound form. Excessive free ACh is known for exhibiting antagonistic effects at other types of cholinergic receptors. Therefore, the antigrowth effects of nicotine are possibly due to its antagonistic effects at the trophoblastic cholinergic receptor directly or through the excessive release of free ACh or both.

The various steps in the working hypothesis of ACh-stimulation and transport coupling are schematically represented in Fig. 2. The data, on which the above scheme is based, were obtained by a number of investigators using in vitro and in vivo experimental models of stimulation-secretion of biogenic amine stimulation-contraction by biogenic amines under a variety of conditions. Not all of these processes are based on firm evidence and some are only tentative. However, this schematic representation provides an outline for studying the sites of action of nicotine in the placenta and its effects on placental transport of nutrients.

1003540336

MATERNAL, PLACENTAL AND FETAL FACTORS WHICH MAY CAUSE POOR FETAL GROWTH:

POSSIBLE SITE OF ACTION OF NICOTINE

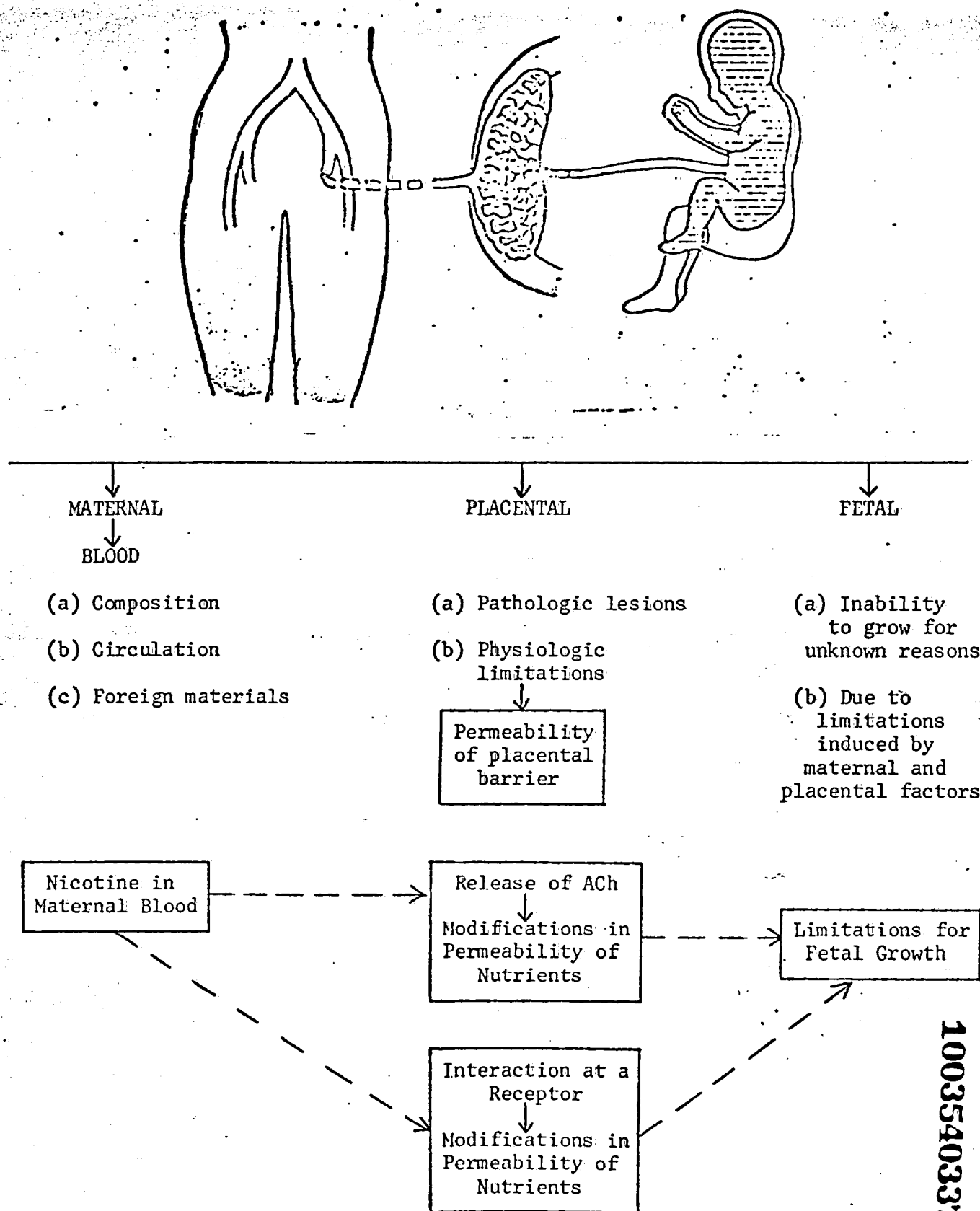


Figure 1

VARIOUS STEPS IN THE WORKING HYPOTHESIS OF ACh-STIMULATION
AND TRANSPORT COUPLING IN THE TROPHOBLAST

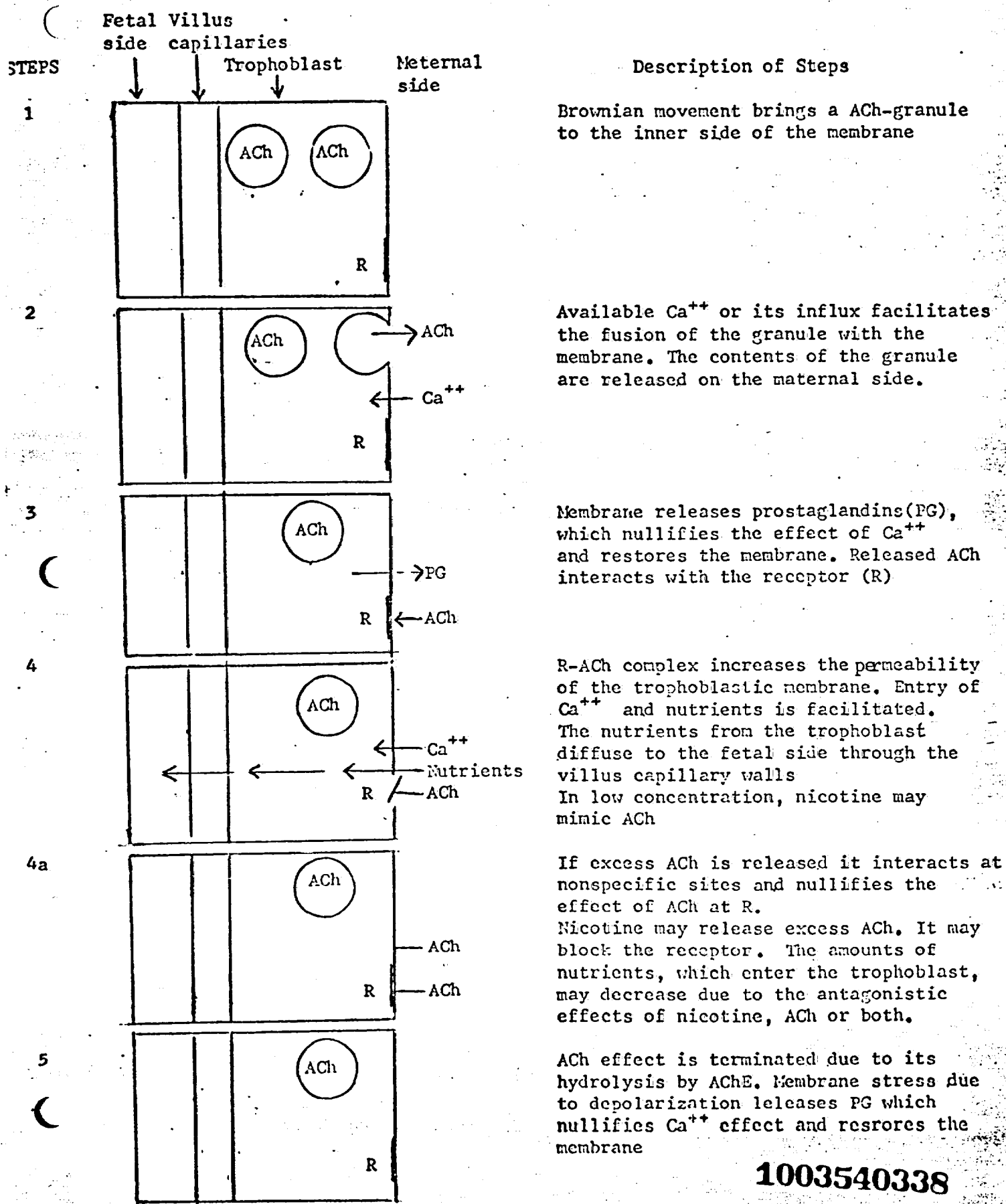


FIGURE 2

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9. Details of experimental design and procedures

Procedures for the collection of human placentae at different gestation periods:

The human placentae were collected from the Vanderbilt University Hospital delivery rooms. During the first trimester, the placentae were collected during therapeutic abortions. During the second trimester, the placentae were collected during spontaneous abortions or premature deliveries. During the third trimester, placentae were collected during natural or therapeutic deliveries. Each placenta after delivery was placed in a plastic bag immediately after delivery and was cooled in an ice bath. The fresh placenta was processed immediately for (1) ACh analysis, (2) experiments involving the perfusion of placental lobes and (3) uptake and release of nutrients, ions and drugs by placental slices and isolated villi. Some placental samples were frozen at -12°C for future analysis of AChE and ChA.

Experimental procedures to study the release of ACh from isolated villi:

It is very easy to tease out villi of the human placenta, providing a considerably less traumatized preparation for in vitro study than the placental slice (19). The trophoblast is exposed to the incubation medium as much as it is to the maternal circulation under normal conditions. We have successfully used such preparations to study the distribution of ACh in human placenta. Such preparations have survived for 6 hours in our laboratories in phosphate or bicarbonate buffers. We have done some initial experiments for the release of ACh into the medium as a factor of time. Our studies on the effects of excess K^+ , Ca^{++} , cyclic AMP, cyclic GMP and prostaglandins on the release of ACh are in progress. We propose to study the effects of nicotine on the release of ACh in the same system.

The isolated villi were incubated for different time periods in an incubation medium (phosphate or bicarbonate buffer) at 37° . The amounts of ACh in the medium and the villi were determined. In a second series of experiments various doses of nicotine (10^{-6} to 10^{-4}M) were added to the medium and again ACh concentrations were determined. From these experiments the net release of ACh into the medium was calculated.

In our preliminary experiments, it was found that ACh was continuously synthesized by ChA and released into the medium. In a third series of experiments, the medium contained a ChA-inhibitor (halogeno-acetylcholines) for preventing the synthesis of new ACh. In a fourth set of experiments the medium contained a ChA inhibitor as well as nicotine. From these two sets of experiments the effect of nicotine on the preformed ACh could be calculated.

In all experiments, the medium contained physostigmine (10^{-6}M) for inhibiting the hydrolysis of ACh by placental AChE.

Experimental procedures for studying effects of nicotine on the release of ACh from ACh-granules of human placenta:

We have indirect evidence that 95% of total human placental ACh is in a bound granular form in human placenta. We propose to isolate these granules using techniques of subcellular fractionation described by De Robertis (20) and Whittaker (21). We propose to study the release of granular ACh using various techniques and treatments described by Whittaker and his collaborators (22,23).

9. Details of experimental design and procedures (cont'd.)

We have used a second approach for the separation ACh-granules from placenta. The trophoblast was separated easily from the trophoblast by dissection. Then the cut trophoblast was subjected to digestion with trypsin and collagenase. Then the contents were subjected to subcellular fractionation, and obtained a rich fraction of ACh-granules. We propose to study the effect of nicotine on these granules.

Experimental procedures for characterization of cholinergic receptors (nicotinic-type or muscarinic type) in placental syntrophoblast:

The working hypothesis assumes the presence of a cholinergic receptor with which ACh or nicotine interacts. Therefore, evidence for the presence of a cholinergic receptor will be obtained.

The binding constants of ^{14}C -ACh and ^{14}C -nicotine to the cholinergic receptors will be determined using the techniques of centrifugal assay and equilibrium dialysis developed by O'Brien and his collaborators (24,25).

The receptor will be characterized as muscarinic or nicotinic by determining the binding constants of specific ^{14}C -labelled agonists and antagonists. Specific agonists: acetyl-1- ^{14}C - β -methylcholine for muscarinic receptors, ^{14}C -nicotine for nicotinic receptors. Specific antagonists: ^{14}C -atropine for muscarinic receptors, ^{14}C -d-tubocurarine or ^{14}C -decamethonium for nicotinic receptors. In receptor protection experiments, the unlabelled agonist should prevent the binding of the labelled antagonist to the receptor. The binding of ^{14}C -labelled agonists and antagonists to the receptors will be verified by the radioautographic procedures described by Waser and his collaborators (26). The binding constants of various agonists and antagonists to cholinergic receptors in tissues other than placenta were determined in the author's laboratory (27,28), with which the binding constants of the cholinergic receptors in human placenta will be compared.

If the human placenta is shown to contain cholinergic receptors, it will become the easily available human source for the isolation of cholinergic receptors. So far cholinergic receptors were not isolated from any mammalian source.

Experimental procedures for studying the transport of nutrients across the placental barrier under the influence of nicotine: perfusion of human placenta:

A method for perfusing a single lobe of human placenta was developed by Schneider *et al.* (29). This system will be used in the present experiments. In this setup, an effective maternal, as well as fetal, circulation was established. Therefore, this system is useful for transport studies. The experimental details and techniques of placental perfusions were described by Schneider *et al.* (29).

The fetal vessels were cannulated and perfusion of fetal circuit was started at a flow rate of 6-12 ml/min at 50-90 mm Hg. Flow rates were checked to make certain that arterial inflow equals venous outflow. This confirmed that an isolated vascular unit has been cannulated and that there was no gross leak across placenta.

Two glass cannulas delivering the maternal perfusion fluid were introduced into the intervillous space through the basal plate. The intervillous space was perfused at a rate of 10-15 ml/min at 80-140 mm Hg. The maternal perfusate

9. Details of experimental design and procedures (cont'd.)

returned from the intervillous space through multiple venous openings in the basal plate and was collected for analysis.

The perfusate was Earle's buffered salt solution containing 4 g% of dextran (low molecular weight) and 100 mg% of glucose. The colloidal osmotic pressure of the perfusate was equivalent to that of normal plasma. The fetal perfusate was equilibrated against a gaseous mixture containing 5% O₂, 5% CO₂ and 90% N₂. The maternal perfusate was equilibrated with 95% O₂ and 5% CO₂. The perfusates were not recycled.

The nutrients, whose placental transport was measured, were added to the maternal circulation and their clearances from the maternal to fetal circulation were measured. The clearances were calculated as follows:

$$\text{clearance (ml/min)} = \frac{(FV-FA)FFR}{MA}$$

where FV = concentration in fetal venous outflow (mg/ml)

FA = concentration in fetal arterial inflow (mg/ml)

MA = concentration in maternal arterial flow (mg/ml)

FFR = fetal flow rate (ml/min)

A series of clearances of a nutrient can be measured before and after addition of various doses of nicotine to the maternal circulation. Using this basic perfusion system, it is possible to study the effects of (a) nicotine, (b) nicotine in the presence of a cholinesterase inhibitor for preventing the enzymatic hydrolysis of ACh which is released by ACh, and (c) nicotine in the presence of a ChA-inhibitor for preventing *de novo* synthesis of ACh on the clearance of selected ions, amino acids, sugars and proteins across the placental barrier.

The following agents are suitable as tools for studying the effects of nicotine on placental transport:

Cholinesterase inhibitors: Physostigmine, neostigmine

ChA-inhibitors: Halogeno-acetylcholine (Iodo-bromo- and chloro-acetylcholines)
N-methyl-4(-1-naphthyl-vinyl) pyridine

Ions: K⁺, Na⁺, Ca⁺⁺

Amino acids: ¹⁴C- α -aminoisobutyric acid

Proteins: ¹³¹I-labelled gamma globulin

Sugars: D-xylose, L-xylose

The specific cholinesterase inhibitors specified above were selected for this study, because their pharmacological effects on cholinergic system, other than that of placenta were well documented. Halogenoacetylcholines have been synthesized by the principal investigator and his collaborators and their pharma-

9. Details of experimental design and procedures (cont'd.)

cology is known (1-3,30-36). N-methyl-4-(1-naphthyl-vinyl)pyridine and its analogs have been introduced by Cavallito and their collaborators as choline acetylase inhibitors (37) and these compounds are available for this investigation from commercial sources.

The ions, K⁺, Na⁺, Ca⁺⁺, were selected for this study due to the following reasons: The placenta is very effective to protect the fetus against potassium depletion (19,38). The reasons for this observation are not known. The maternal hypokalemia is not reflected in the fetal blood or tissues. With sodium deprivation of the mother, various degrees of hyponatremia are induced in the fetus of the rat or the sheep (39,40). The results are variable and are not conclusive as to whether the placenta protects the fetus against sodium deprivation (19,40). In human placenta, there is a relationship between ²⁴Na transfer and gestational age (41). The transfer rate increases until 36 weeks of gestational age and then decreases at term. A fall off in placental efficiency towards term is consistent with the clinical concept of post-maturity (38). In our experiments fluxes of ²⁴Na and variations in ACh concentrations will be measured simultaneously within the same placental isolated villi. Ca⁺⁺ is included in this because of its importance to fetal growth and its possible role in the secretion of ACh.

Placenta concentrates ¹⁴C- α -aminoisobutyric acid from maternal circulation against a concentration gradient and releases on a downhill gradient to fetus (42). As this amino acid is non-metabolizable, it is suitable to study the effects of cholinergic agents on the placental transfer of amino acids. It is of interest to note here that a cholinergic mechanism has been implicated as a fine control in regulating the production of protein in spiders (43).

One of the factors in the protection of the newborn is the antibodies received passively from the mother, and the mechanisms for this transfer are not known. The investigations of Dancis et al. (44) indicate that the route for the transfer of proteins from mother to fetus is allantoic placenta. This transfer of proteins is significant in the early pregnancy and in late pregnancy in the rat (45), monkey (46), and human (44). In the above studies on protein transfer ¹³¹I-labelled gamma globulin has been used. In the experiments of the above design the protein-bound radioactivity represents transferred protein (47). Therefore, ¹³¹I-labelled gamma globulin will be used to measure placental transfer of proteins during manipulation of placental cholinergic system by nicotine.

D-xylose, which is transferred through the placenta at 3 times the rate of L-xylose, is a nonmetabolizable model for glucose. Therefore, the placental transfer of D-xylose will be measured during the manipulation of cholinergic system by nicotine (19,48).

Analytical Methods

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Assay of ACh in placental tissues by gas chromatography:

A representative sample of placenta is cleared of blood clots, and is homogenized in a Sorvall Omnimixer containing acetonitrile and trichloroacetic acid (1.0 ml/0.1 g wet tissue, TCA 2%). The homogenate was centrifuged and the precipitate was rejected. The supernatant was diluted with equal volume of distilled water and the resulting solution was extracted twice with equal volumes of diethyl ether and the ether layer was discarded. A gentle stream of nitrogen gas was

9. Details of experimental design and procedures (cont'd.)

bubbled through the aqueous layer to remove the residual ether. The ether-free aqueous layer (2.0 ml) was diluted with equal volume of distilled water. To the resulting solution tetramethylammonium iodide (6.0 μ g) and KI-I₂ solution (0.15 ml) were added. The reaction mixture was centrifuged and the supernatant was aspirated. The precipitate of quaternary ammonium compounds was subjected to gas chromatography using pyrolysis techniques (49,50) or Jenden's reaction (51,52).

This method is schematically represented in Figure 3.

Analysis of tissues for cholinesterases:

Three different types of methods are routinely used in our laboratories for the assay of cholinesterases. In all three methods ACh was used to give total cholinesterases. Acetyl- β -methylcholine was used as a specific substrate for AChE because it was not hydrolyzed by pseudo-ChE (53). Butyrylcholine was used as a substrate for the analysis of pseudo-ChE (54). It is a very weak substrate of AChE. However, a correction factor has been developed for the hydrolysis of butyrylcholine by AChE for the assay of pseudo-ChE (55). Each method is applicable for a specific situation based upon the amount of tissue available and the sensitivity required in the assay. In all three methods tissue homogenates were used as the source of the enzymes.

In the first method, the hydrolysis of choline esters were determined by titrating the acids released during their hydrolysis using a Radiometer pH-stat titration unit. The details were described in the papers published in the literature (56).

The second method is a differential radiochemical assay for AChE and pseudo-ChE using ¹⁴C-substrates (55). The differential rates of hydrolysis of 1-¹⁴C-acetyl- β -methylcholine (pS 2.5) and 1-¹⁴C-butyrylcholine (pS 2.5) were used to estimate AChE and pseudo-ChE activities in tissues. The tissue homogenate and the ¹⁴C-substrate were incubated for varying periods (0-50 min). Initial linear velocities were obtained from rates of hydrolysis during 0-10 min. Acetyl- β -methylcholine was not significantly hydrolyzed by pseudo-ChE, while butyrylcholine was hydrolyzed by AChE at 12% of the rate of acetyl- β -methylcholine. Therefore, a correction was applied in the estimation of pseudo-ChE by butyrylcholine. This method is of special value in estimating cholinesterases in small amounts of tissues (plasma 0.01-0.1 ml, tissue, 10 mg). This method has been used successfully for the estimation of cholinesterases in the human placenta.

In a third method, ¹⁴CO₂ produced from the interaction between NaH¹⁴CO₃ and acid products of esters hydrolyzed by cholinesterases were trapped with 2-phenethylamine and counted with a liquid scintillation counter.

Experiments were done using a Warburg apparatus with 0.4 ml of 2-phenethylamine placed in the central well of the flask to trap ¹⁴CO₂ produced. The main compartment of the 15 ml Warburg flask contained 2.5 ml of modified Krebs-Ringer bicarbonate buffer with 1/10 the normal concentration of NaH¹⁴CO₃ and a radioactivity level of 2.22×10^5 dpm (2.3×10^{-3} M NaH¹⁴CO₃, 7.5×10^{-2} M KCl, 7.5×10^{-2} M NaCl, 4×10^{-2} M MgCl₂). Tissue samples containing the enzyme (0.2 ml) were added to the main compartment of the flask. The side arm contained 0.3 ml of substrate (final concentration of 10^{-3} M) dissolved in the modified buffer which contained no radioactivity. The Warburg flasks were shaken for 15 minutes before the substrate (ACh, acetyl- β -methylcholine, or butyrylcholine) was dumped

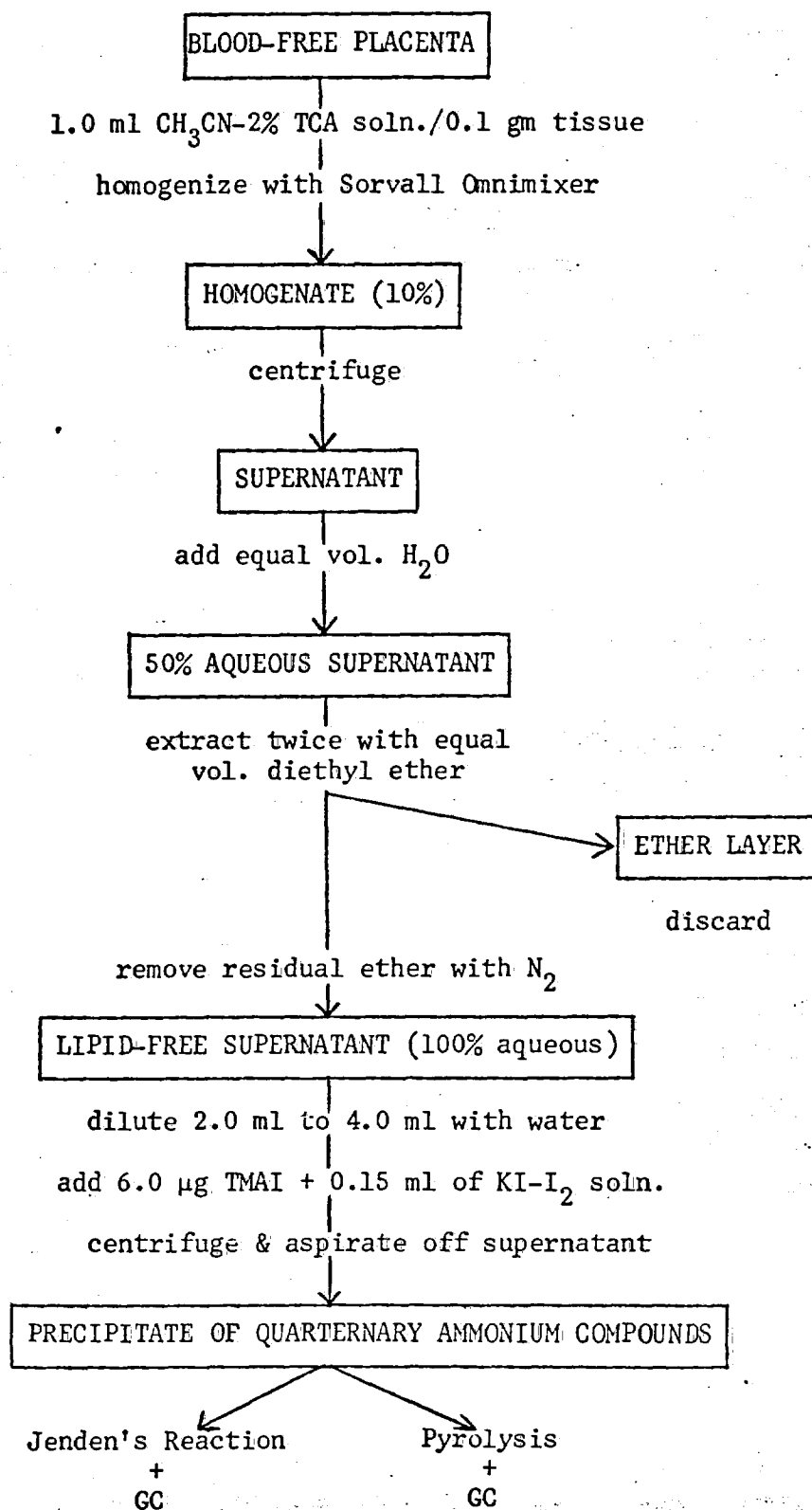


Figure 3: The 5 step procedure includes: (a) Extraction with CH₃CN and tri-chloroacetic acid, (b) Centrifugation to remove insoluble membranes, (c) Removal of lipids with ether, (d) Precipitation of quaternary ammonium compounds with potassium iodide, iodine and tetramethylammonium iodide, (e) Subjection of quaternary ammonium compounds to pyrolysis or Jenden's reaction.

9. Details of experimental design and procedures (cont'd.)

into the main compartment. The reaction mixture was shaken for various periods of time and the 2-phenethylamine with ^{14}C was transferred to a counting vial containing 10 ml of scintillation cocktail (2,5-diphenyloxazole, 7 g.; naphthalene, 100 g.; dioxane, 1000 ml). The central well was washed twice with 2×0.3 ml of methanol and the washings were added to the scintillation cocktail and counted with a liquid scintillation counter. Further details of the method were described by Sastry and White (53) and Sastry and Chiou (57). This method was very useful to determine cholinesterases bound to membrane fractions.

Analysis of choline acetyltransferase (ChA) placental tissues:

ChA in placental tissues was assayed by the formation of acetyl- ^{14}C -choline from choline and acetyl- ^{14}C -CoA. The details for the separation of ^{14}C -ACh from the reaction mixture and counting of the samples were described in the papers by the author and his collaborators (1-3), and McCamen and Hunt (58). We used this method in the purification of ChA from the human placenta (1,2) and the rat brain (3).

Analytical methods for important ions: Conventional analytical methods with necessary modifications will be adopted. The following methods were listed as examples:

- (a) Sodium and potassium: Emission flame spectrophotometry
- (b) Chloride: Automatic chloride titrator (59)
- (c) Calcium: A micromethod developed by Geyer and Bowie (60) is routinely used in the author's laboratory to determine the concentrations of calcium in biological samples. The method is based on emission flame spectrophotometric techniques. The final samples contain isopropyl alcohol, water and known concentrations of sodium and potassium chlorides. Isopropyl alcohol is used to increase the luminosity. Sodium and potassium chlorides are employed to control the normal interference from sodium and potassium. Samples are analyzed employing oxygen-hydrogen flame at a wave length of 422.66 m μ with a slit width of 0.04 m μ . The red filter is employed in conjunction with the photomultiplier. Calcium values less than 2 $\mu\text{g/ml}$ are readily analyzed by this method. The author's laboratory contains a Beckman DU-2 spectrophotometer equipped with flame attachment, spectral energy recording adaptor and a recorder.

Radiometric methods:

The isotope ions, ^{24}Na and ^{42}K , used in the present investigation can be measured by gamma scintillation spectrometry using a multi-channel analyzer. ^{45}Ca will be assayed by beta counting methods. Gamma scintillation spectrometric methods have been used in authors laboratory during the last 10 years, and typical examples are described in author's papers (61,62).

^{14}C -compounds will be assayed using liquid scintillation methods. Typical examples are described in author's papers (1-3) and the papers by Harbison and his collaborators (63,64).

10. Space and facilities available

Total space (2 laboratories, 1 office, 1 instrument room) available for Dr. Sastry consists of 936 square feet, equipped with modern conventional laboratory facilities suitable for chemical, radio-biological and animal work. These facilities contain a roomette (which has separate vents for removal of odors) for animal surgery and isolation. These laboratories contain built in refrigerators (3), radiation hoods (2), and a walk-in hood for chemical syntheses.

The human placentae were collected from the delivery rooms and surgical facilities of Vanderbilt University Hospital with the help of the staff of the Department of Obstetrics and Gynecology. The Department of Pharmacology, the Department of Obstetrics and Gynecology, and the delivery and surgical rooms of Vanderbilt University Hospital are located within the same building complex. The collection and transport of placenta after delivery takes less than 5 minutes from the delivery or surgical rooms to the Department of Pharmacology.

A number of items of equipment are available in the author's laboratory to perform unit processes for conducting various steps in this research.

1) For chemical and physical manipulations:

- a) Fisher-roll around vacuum system
- b) Bendix Automatic Polarimeter
- c) Precision Scientific-Lotemptrol Circulating Unit
- d) Welsh vacuum pump

2) For chromatographic identification:

- a) Misco-Electrochromatography Unit
- b) Precision-Scientific-Chromac for circular paper chromatography
- c) Equipment for ascending, descending and circular paper chromatography

3) For analytical procedures:

- a) Aminco-respiration apparatus with accessories
- b) Radiometer pH-stat titration unit equipped with a pH meter, a recorder and an autoburette
- c) Beckman DU-Spectrophotometer (with flame attachment)
- d) Corning Model-12 Research pH Meter
- e) Nuclear-Chicago Autogamma system with a 512 channel analyzer
- f) Mettler-semimicro and macrobalances
- g) Cahn Electrobalance (capacity, 1000 μ g)

4) For bioassay procedures:

- a) Harvard Kymograph
- b) Gilson Polygraph and accessories (4 channels)
- c) Narco-Biosystems Physiograph-desk model DMP 4A (4 channels)
- d) Isometric and isotonic transducers (4 each)
- e) Phipps & Bird-Isolated organ tissue baths (2)
- f) Grass 88 stimulator
- g) Harvard continuous infusion and peristaltic pumps
- h) Harvard respirators for dogs, rats and other small animals

10. Space and facilities available (cont'd.)

5) For statistics:

Marchant electronic calculators, Cogito 516 & 1016 PR

6) Equipment in the Department of Pharmacology available for part time for this project:

- a) Beckman Model L Ultracentrifuge
- b) Sorvall-Superspeed Automatic Refrigerated Centrifuge
- c) Beckman DK Recording Spectrophotometer
- d) Farrand Spectrofluorometer
- e) Packard Tricarb Liquid Scintillation Spectrometer (Model 3375)
- f) Olivetti Underwood Programmer, Model 101
- g) Nuclear Chicago 512-Channel Gamma Scintillation Spectrometer equipped with multiregion-interest-accessory, sample changer (capacity) and teletype
- h) Hewlett-Packard Model 5750 gas chromatograph

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel: Attached Separately

13. Publications: (five most recent and pertinent of investigator(s)):

- 1) Molecular Aspects of the Interactions of Halogeno-acetylcholines with Cholinesterase, B.V. Rama Sastry and Chung Y. Chiou, Biochim. Biophys. Acta, 167, 339-354, 1968.
- 2) Cholinergic activities of halogeno-acetylcholines. Chung Y. Chiou and B.V. Rama Sastry, J. Pharmacol. Exptl. Therap. 172: (2) 351-366, 1970.
- 3) Dissociation constants of D and L -lactoylcholines and related compounds at cholinergic receptors. H.C. Cheng and B.V. Rama Sastry, J. Pharmacol. Exptl. Therap., 180: 326-339, 1972.
- 4) Kinetic mechanisms of human placental choline acetyltransferase. B.V. Rama Sastry and G.I. Henderson, Biochem. Pharmacol. 21: 787-802, 1972.
- 5) Stereoisomerism and drug action in the nervous system. B.V. Rama Sastry (Invited Author), Ann. Rev. Pharmacol. 13: 253-267, 1973.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

B.V. Rama Sastry, Ph.D., Professor of
Pharmacology

40%

\$ --0--

Technical

Lillian K. Owens, Research Assistant

100%

\$ 7,878

Fringe Benefits

1,123

Sub-Total for A \$ 9,001

B. Consumable supplies (by major categories)

Chemicals and Drugs

\$ 500

Glassware and Plasticware

500

Surgical Supplies

100

Sub-Total for B \$ 1,100

C. Other expenses (itemize)

Travel: To attend national meeting to present work
supported by this grant

\$ 300

Other: Expenses involved with obtaining human placenta

300

Sub-Total for C \$ 600Running Total of A + B + C \$ 10,701

D. Permanent equipment (itemize)

Minor items of equipment related to
the gas chromatographic analysis of
acetylcholine

\$ 500

Sub-Total for D \$ 500

E. Indirect costs (15% of A+B+C)

E \$ 1,605Total request \$ 12,806

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | \$9,457 | \$1,100 | \$700 | \$-0- | \$1,689 | \$12,946 |
| Year 3 | \$9,977 | \$1,200 | \$700 | \$-0- | \$1,782 | \$13,659 |

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C.K.
R.C.H.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

| CURRENTLY ACTIVE | | | |
|--|--------------------------------|----------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| "Biological Significance of Choline Esters Other than ACH" | NIH-NS04699 | \$42,544 | 9/1/73-8/31/74 |
| | | 44,822 | 9/1/74-8/31/75 |
| | | 47,303 | 9/1/75-8/31/76 |

| PENDING OR PLANNED | | | |
|--|--------------------------------|-----------|--------------------|
| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
| "Cholinergic System, Placental Transport and Fetal Growth" | NIH HD08561 | \$311,746 | 5/1/74-4/30/79 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Vanderbilt University

Mailing address for checks Mr. Paul Gazzerri, Jr.,
Associate Vice Chancellor for Medical Affairs
Operations and Fiscal Planning
Vanderbilt University Medical Center
Nashville, Tennessee 37232

Principal investigator

Typed Name B.V. Rama Sastry, D.Sc., Ph.D.

Signature B.V. Rama Sastry Date Nov 20, 1973

Telephone 615 322-2207 N/A
Area Code Number Extension

Responsible officer of institution

Typed Name Allan D. Bass, M.D.

Title Associate Dean, Biomedical Sciences

Signature [Signature] Date 11/26/73

Telephone 615 322-2281 N/A
Area Code Number Extension

#969-TIMASHEFF

1003540350

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1974

Grant application No. 969

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Jacobson,
Sommers

Subject: Professor Serge N. Timasheff, Ph.D., Brandeis University
New application No. 969
"Interactions of Microtubules with Nicotine and Nicotine
Products"

History

A preliminary inquiry concerning this proposal was handled as Case No. 257: the Executive Committee voted to encourage formal proposal.

Request

Application No. 969 requests \$52,450 plus two additional years.

Document Submitted

Attached is application dated January 25, 1974 (22 pages).

F.W.N.
F.W.N.

FWN:wg
Encl.

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ack
4/3/74
gh

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date:

January 25, 1974

1. Principal Investigator (give title and degrees):

Professor Serge N. Timasheff, Ph.D.

2. Institution & address:

Brandeis University
Waltham, Massachusetts 02154

3. Department(s) where research will be done or collaboration provided:

Graduate Department of Biochemistry

4. Short title of study:

Interactions of microtubules with Nicotine and Nicotine Products

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

It is proposed to undertake a detailed study of the mechanism on the molecular level of binding of nicotine and products of nicotine metabolism to tubulin, the subunit protein of microtubules, and of the effects of this complexation on the self-assembly of the microtubule protein subunits which leads to microtubule formation. Specifically, the interactions of tubulin with nicotine, its liver metabolites and chemical derivatives will be examined quantitatively by column chromatographic and spectroscopic techniques. The self-association of tubulin in the presence of these ligands will be examined by sedimentation, light scattering, calorimetric and column chromatographic techniques. The results will be analyzed in terms of most modern rigorous thermodynamic theory. Furthermore, the effect of these tobacco products on the morphology of microtubules will be studied by electron microscopy, and their interference with the self-assembly of these organelles will be followed by in vitro microtubule reconstitution experiments. Such knowledge will advance the understanding of the mechanism by which nicotine affects biological processes. It is only with such an understanding that a rational approach can be made eventually to the question of the prevention of harmful action.

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While nicotine is known to have strong and complex pharmacological effects both on the central and the peripheral autonomic nervous systems, its mechanism of action at the molecular level is not understood (1,2). Recently, it has been found in our laboratory that nicotine binds strongly to tubulin, the protein subunit of microtubules (3). Microtubules have been identified in recent years as key components of most cells (4,5,6). They are found in the axons, dendrites and neurites of nerve cells (7,8,9) and have been implicated in sensory transduction (10); they form part of the mitotic spindles (11,12,13) of dividing cells, and they are involved in maintenance of cell shape and in the intracellular transport of material (14,15). Microtubules are not static entities, but they are in dynamic equilibrium with subunits in the cytoplasm (16,17,18,19), being assembled as required by cell function. The structure of microtubules is known to be perturbed or destroyed by complexation with ligands, such as various plant alkaloids (20,21,22), with the concomitant destruction of physiological function. In view of these facts, it seems of importance to pursue in depth our observation of the binding of nicotine to microtubule subunits and to establish the detailed mechanism of this binding and

9. Details of experimental design and procedures (append extra pages as necessary)

the nature of related effects on the structure of microtubules and their ability to self-assemble. The gaining of such understanding should significantly advance the elucidation of the molecular mechanism by which nicotine interacts with tissues to produce its various pharmacological effects.

9. Details of experimental design and procedures

The proposed experimental work will consist of the following studies:

1. Binding of nicotine, nicotine metabolites and derivatives of nicotine to tubulin;
2. Effects of nicotine and nicotine-related compounds on the self-association properties of tubulin;
3. Effects of nicotine and nicotine-related compounds on the structural stability of tubulin.

The investigation will be done using calf brain microtubule protein. This is an excellent system for study, since fresh calf brains are readily available, and the isolation procedure for obtaining homogeneous protein is well established (23,24). The purified calf brain microtubule protein will be stored in 1M sucrose, according to the method developed in this laboratory by Frigon and Lee (25) who have demonstrated that this medium stabilizes this protein, which normally is highly unstable and readily denatured. A further advantage of using calf brain microtubule protein is that it has been recently characterized in great detail by Lee et al. (24,26) of this laboratory.

In view of these ground-breaking studies on the protein, this laboratory is at present in a unique position for carrying out the proposed studies.

Lee et al (24) have shown that the chemical composition (amino acid sequence) of calf brain microtubule protein is similar to that of microtubule subunits from other sources. The protein consists of two almost identical subunits, each with a molecular weight of 54,000 + 1,000. There is one intrachain disulfide bridge and several free

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() sulfhydryl groups fully accessible to solvent. The native isolated protein consists of dimers with a molecular weight of 110,000. Weisenberg and Timasheff have shown that, in the presence of magnesium, these dimers self-associate to large species (23) with molecular weights in the vicinity of 2.5 million, as found by Frigon and Timasheff (27). This species is similar in size to the disk-like structures which have been observed (28) and proposed as centers of nucleation for the self-assembly of microtubules.

The assembled microtubules are hollow, usually straight cylinders, approximately 240A in diameter. The outer walls are 50A thick and are composed of the tubulin subunits. The assembled microtubules, in vivo, are in a state of dynamic equilibrium with a soluble pool of subunits (18,29). Thus, it appears that these structures are assembled as needed by cellular function.

Binding of ligands to microtubules strongly perturbs or destroys their structure, with a resulting loss of their physiological function. This effect has been studied in particular detail for the plant alkaloids colchicine and vinblastine, which not only destroy these structures in vivo (20,22), but also strongly affect the self-association of tubulin in in vitro solution studies, as shown by Weisenberg and Timasheff (23).

() The outlined studies will be carried out by a variety of protein physico-chemical and chemical techniques. These include equilibrium and velocity sedimentation, light scattering, gel filtration, circular dichroism, fluorescence, ultra-violet and infra-red spectroscopy, microcalorimetry, densimetry, controlled chemical modification of protein side chains, potentiometric titration, in their various ramifications as required for each particular study, and with the rigorous quantitative application of the most modern theories in the analysis of the results. These methods, which have been used with a great deal of success in the past in the principal investigator's laboratory, are described well in the review literature, for example in three volumes recently edited by the principal investigator (30) (Tables of contents enclosed.). These techniques do not need to be described in detail here. Suffice it to remark that the criterion used in the past by the principal investigator will be applied to these studies, as well; namely that results obtained on a problem by several unrelated techniques should be quantitatively consistent.

Binding of Nicotine and Nicotine-Related Compounds to Tubulin

Nicotine is actively metabolized by man and other mammals by a variety of pathways to give a number of products (31,32). The principal pathways and products are summarized in Figure 1. It is not clear at present, however, what role the products of nicotine metabolism play in the physiological responses to nicotine (32,33).

() Since it has been observed in our laboratory that nicotine binds strongly to tubulin, studies of the mechanism of the binding of nicotine to tubulin will be pursued in detail. These will be supplemented

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by similar binding studies of products of nicotine metabolism and other nicotine-related compounds. Initially, the binding of nicotine metabolites to tubulin will concentrate on cotinine and cotinine-N-oxide, since their incorporation in even minute amounts into microtubules will be detectable by radioimmunoassay techniques developed for these compounds, as well as for nicotine, by Dr. H. Van Vunakis of this Department (34,35). In later stages of the investigation, similar studies will be extended to other metabolites. The compounds in question are either available commercially or can be synthesized according to methods described in the literature (35).

In order to establish the mechanism of binding of a ligand to a protein it is essential to determine the stoichiometry of the association and the variation of the equilibrium constant with changes in conditions (e.g. temperature, pH of the medium, nature and concentration of salt in the buffer system, etc.) (36). From the thermodynamic parameters obtained in this way, namely the free energy (ΔG), the enthalpy (ΔH), the entropy (ΔS) and the heat capacity (ΔC_p), and from the patterns of their variations with changes in environment, the nature of the forces operative, and thus of the types of interactions and of the chemical groups involved, can be deduced. These conclusions can be confirmed and further refined by proper chemical modifications of the protein and the ligand followed by binding measurements. For example, groups identified on the protein by thermodynamic measurements can be selectively blocked or altered; on the other hand, groups on the ligand can be modified by the synthesis of the proper derivatives, and the component parts of a complex ligand, such as nicotine, can be studied separately (in the case of nicotine this would involve separate binding studies of pyridine and of N-methylpyrrolidine); such experiments would determine the contribution of each ring of nicotine to the total binding.

The binding studies will be performed using a variety of techniques, selected according to the properties of the individual ligands and to the particular piece of information sought in a given experiment. These will include equilibrium dialysis, ultrafiltration, absorption spectrophotometry, fluorescence and various column methods (e.g., the one described by Hummel and Dreyer (37), and the column scanner developed by Ackers (38)). By applying these experimental methods to a given protein-ligand system, the number of binding sites for the ligand on the protein and the intrinsic binding constant can be determined. By making experimental measurements at several different temperatures, the van't Hoff enthalpy and also the entropy of binding will be calculated. In addition to the equilibrium studies, direct calorimetric determinations of reaction heats will be performed.

As stated above, in order to arrive at an exact understanding of the mechanism of interaction, it is necessary to sort out

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the various types of forces which contribute to the binding.

The interactions between biological molecules involve many complex reactions of covalent and noncovalent forces, such as dipole-dipole interactions, hydrogen bonds, hydrophobic and Coulombic interactions. These may be identified and sorted out by subjecting the associating system to various probes and monitoring the response of the system. The thermodynamic characteristics of the various types of non-covalent interactions and the probes that can be used to distinguish between them are summarized in Table I (39,41).

TABLE I
Intermolecular Forces

1. Electrostatic

| | | | |
|------------------|------------------|------|--|
| a. Non-specific | ΔG° | >0 | Vary pH and ionic strength |
| b. Specific site | ΔG° | <0 | Vary pH and ionic strength |
| | ΔH° | <0 | Chemical modification of site |
| | ΔS° | >0 | Titration of specific groups |
| | | | Vary nature of supporting electrolyte ($F^- < Cl^- < ClO_4^-$) |

2. H-Bonding

| | | |
|------------------|------|-----------------------------|
| ΔG° | <0 | Vary temperature |
| ΔH° | <0 | Identify groups by chemical |
| ΔS° | <0 | and spectroscopic means |

3. Hydrophobic

| | | |
|------------------|------|------------------------------|
| ΔG° | <0 | Vary temperature |
| ΔH° | >0 | Vary medium (D_2O , |
| ΔS° | >0 | Hofmeister series: |
| ΔC_p | <0 | $SCN^- < Cl^- < SO_4^{2-}$) |

4. van der Waals

Small

In probing intermolecular interactions, a particularly valuable approach is through variations of the medium. By altering the polarity of the medium, such as by addition of organic solvents, the dielectric constant of the medium would be decreased. This strengthens the electrostatic interactions, but weakens the hydrophobic interactions. Another sensitive probe is available in the Hofmeister series of anions (142). In general, the anions may be listed as a series in increasing degrees of strengthening hydrophobic interactions: SCN^- , ClO_4^- , Br^- , Cl^- , Ac^- , F^- , HPO_4^{2-} , SO_4^{2-} . It can be expected, however, that their effect on ion pair formation should be just the opposite (143).

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The pH-dependence of the binding will be followed. This will give information on ionizable groups involved in the process. Changes in the state of ionization of microtubule protein during interactions can also be followed calorimetrically. In buffered aqueous solutions, the observed calorimetric heat, Q_{obs} , is given by the relation

$$Q_{obs} = Q_{rx} + n_H Q_{ion}$$

where Q_{rx} is the heat of the reaction, Q_{ion} is the heat of ionization of the buffer, and n_H is the number of protons involved. If a reaction is studied at constant pH in buffers of different heats of ionization, then n_H AND Q_{rx} may be readily determined. The sign associated with n_H indicates whether protons are released or absorbed during the reaction. This powerful method has been used in a number of studies (44,46) and has revealed the presence of reactive protons not previously suspected.

Following the studies with nicotine itself, similar measurements will be carried out with pyridine and N-methylpyrrolidine, as well as with chemical derivatives of nicotine, with chemical modifications dictated by the conclusions of the thermodynamic studies.

Effects of nicotine and nicotine-related compounds on the self-association properties of tubulin.

The tubulin-nicotine binding studies will be paralleled by studies of the perturbation of the self-association of tubulin to form microtubules. Since normal intact microtubules are required for their physiological function, a perturbation of the morphology of these structures or in the pathways of their assembly results in serious perturbations of biological function.

Studies in the principal investigator's laboratory indicate that, in the presence of magnesium ions, tubulin self-associates in steps to a species that, in size, is similar to the discs that have been reported as precursors of microtubule formation. Therefore, the effect of nicotine binding on tubulin self-association in the presence of magnesium will be investigated, in a manner similar to the studies in the principal investigator's laboratory of the perturbation of magnesium-induced association of tubulin by the alkaloid, vinblastine (23).

The association studies will be pursued primarily by sedimentation (47-55) and light scattering (56-60), since the data obtained by any one technique alone may not be sufficient to elucidate clearly the behavior of the system. The initial investi-

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gations will be performed by sedimentation velocity. The dependence of the sedimentation coefficient and of the area distribution under the reaction boundary on both protein and nicotine concentrations will be determined. The results of this study will be analyzed in terms of the theory of sedimentation for ligand-mediated association of Cann and Goad (161) and the simulation of sedimentation patterns of associating systems, as described, for example, by Gilbert and Gilbert (155). The molecular weight distribution as a function of protein concentration will be obtained from sedimentation equilibrium studies. This will enable the calculation of the equilibrium constant of this system. Measurements as a function of temperature will yield the van't Hoff enthalpy, and, therefore, the entropy of the system. Furthermore, microcalorimetric studies of this association process, as well as of dilution-induced dissociation of the aggregates (44), will provide additional valuable thermodynamic information for furthering our understanding of these processes.

Following the indications of Table 1, the effects of ionic strength, specific anions, D_2O and non-polar solvents on the protein interactions in the presence of nicotine will also be investigated. The interpretation of such measurements may require the application of multicomponent theory to include the consideration of preferential interaction of solvent components with the protein (62,63).

Following the studies with nicotine itself, similar measurements will be carried out with nicotine derivatives, following as a guide the results of the binding studies described above.

As knowledge develops on the tubulin-nicotine interaction, the solution studies will be extended to the level of self-assembly of microtubules in vitro. Reconstitution experiments will be carried out according to the method of Weisenberg (64). The system will contain in addition, ^{nicotine} or a related compound identified through solution studies as a perturber of tubulin self-association. It is expected that such perturbation on the molecular level should have a strong effect on the reconstitution of microtubules and on the morphology of assembled structures, if such are formed in the presence of the ligands.

The results of these experiments should permit to carry out well controlled in vitro experiments on the effects of self-assembly perturbing nicotine-related ligands on reconstituted microtubules. In these experiments, microtubules will be reconstituted according to the method of Weisenberg (64), ligand will be added to the system and a kinetic study of microtubule degeneration will be carried out by changes in light scattering and in details of structures observed by electron microscopy. In the late stages of microtubule degeneration, structures sufficiently small to be followed by solution

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thermodynamic and conformational methods could be formed. This would complete the cycle of experiments. In this manner, it is expected that a direct link should be established between the quantitative solution studies on the molecular level and the morphological effects.

Effects of nicotine and nicotine-related compounds on the structural stability of tubulin.

Since the binding of ligands to a protein may induce conformational changes, the thermodynamic studies will be complemented by careful conformational examination of the various systems. These experiments will be done using the methods of circular dichroism and fluorescence and, if necessary, NMR. Preliminary experiments in the principal investigator's laboratory indicate that, in the near ultraviolet region, some rotational bands are perturbed in the circular dichroism spectrum by the binding of the alkaloid vinblastine. Similar perturbations may be expected to accompany the binding of nicotine. While the affected bands are most likely due to tyrosine transitions, their identification will be carried out by controlled chemical modification of the tyrosines by acetylation and cyanuration (65), followed by binding experiments and circular dichroism and fluorescence measurements.

Furthermore the effect of nicotine binding on the structural stability of tubulin will be examined by parallel thermodynamic, conformational and activity experiments as a function of ligand composition. The thermodynamic experiments will be aimed at the determination of the transition temperature of tubulin. The transition temperature will be determined by monitoring the change in UV absorption in the 280 nm region and treating the results by standard thermodynamic methods. Densimetry measurements will also be carried out. These will result in a measurement of the volume change, ΔV , which occurs when nicotine is bound to tubulin, since (66)

$$\Delta V = M_2 [\bar{v}_2 \text{ (in the presence of nicotine)} - \bar{v}_2 \text{ (in water)}]$$

where M_2 is the molecular weight of the protein.

The thermodynamic information available from the transition temperature measurements, supplemented by microcalorimetric measurements, on the effects of nicotine on the stability of the tubulin molecule, together with the knowledge of the energy of stabilization of the system by the ligands and of the change in apparent volume of the protein obtained from the densimetry measurements, should lead to a further understanding of the nature of the interactions and of the manner in which nicotine and related compounds affect the stability of the tubulin molecule.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Dr. Serge N. Timasheff, Prof.
and Principal Investigator

15

-

Dr. Marina Gorbunoff, Senior
Research Associate

20

\$ 3,600

Dr. James Lee, Sr. Research Asso.

20

-

Dr. Donald Atha, Research Asso.

100

9,500

To be appointed: Grad. Research Fellow

100

3,500

Technical

Valda Bolis, Research Technician

100

8,000

Miriam Torney, Adm. Assistant

20

1,500

Staff: Dishwasher

20

1,250

Fringe Benefits

2,188

Sub-Total for A 29,538

B. Consumable supplies (by major categories)

Glassware

1,000

Chemicals and Biochemicals

2,000

Chromatographic Supplies

1,500

Replacement cells, Xenon lamps and liquid nitrogen
for Cary 60 Spectropolarimeter

1,500

Sub-Total for B 6,000

C. Other expenses (itemize)

Travel to pertinent meetings

1,000

Publication costs

1,000

Servicing of equipment

900

Xeroxing and drafting costs

500

Sub-Total for C 3,400

Running Total of A + B + C 38,938

D. Permanent equipment (itemize)

LKB Fraction collector UVcord column monitoring
device, recorder and other accessories

7,000

Replacement drives and rotors for analytical
ultracentrifuge

1,000

Sub-Total for D 8,000

E. Indirect costs (15% of A+B+C)

E 5,512

Total request 52,450

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|--------|
| Year 2 | 31,015 | 7,000 | 3,600 | 1,000 | 6,242 | 48,857 |
| Year 3 | 32,536 | 7,500 | 3,800 | 1,000 | 6,575 | 51,411 |

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X.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|-------------------------------------|-----------|--|
| Structure and Interactions of Proteins in Solution | NIH-GM 14603 | \$236,538 | 9/71-8/31/76 (current year: \$37,564) reduced from \$44,192; subsequent years equally reduced. |
| Thermodynamics and Geometry of Protein Interactions | NSF-GB 38544X | \$ 90,000 | 6/1/73-5/31/76 (Total cost for 3 years) |
| *The Interaction of Brain Microtubule Protein with Vinblastine and Other Anti-Cancer Drugs | American Cancer Soc. Mass. Division | \$ 12,750 | 1/1/74-12/1/74 <u>This grant is limited to one year and cannot be renewed.</u> |

*Largely to support one postdoctoral research associate for one year.

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--------------------------------|-----------------------|--------------------|
| Tubulin Associations and effects of anti-cancer drugs | NIH- decision pending | \$63,526 REQUESTED | 11/1/74-10/31/75 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name SERGE N. TIMASHEFF
 Signature Serge N. Timasheff Date Jan. 28, 1974
 Telephone 617-647-2720
 Area Code Number Extension

Checks payable to

Bernard I. Levinson
 Coordinator of Sponsored Research
 Mailing address for checks
Brandeis University
Waltham, Mass. 02154

Responsible officer of institution

Typed Name Marver H. Bernstein
 Title President
 Signature Marver H. Bernstein Date 1/21/74
 Telephone 617-647-2201
 Area Code Number Extension

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#943 - VOLLE

1003540362

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

October 22, 1973

Grant application No. 943

PHARMACOLOGY

To: The committee comprising Drs. Bing, Jacobson, and Liebow

Subject: Robert L. Volle, Ph.D., University of Connecticut, Farmington
New application No. 943
"Molecular Mechanisms Underlying the Development of Tolerance
to Nicotine"

History

This proposal was case #214, and application was encouraged.

Dr. Volle has AMAERF support, 1972 - 1974, now terminal. Publications from studies under this support appear in his bibliography in the application.

Request

Application #943 requests \$22,028.00 plus two additional years.

Document Submitted

Attached is a copy of application dated 9/7/73.

FWN:gh

Enclosure

JMM

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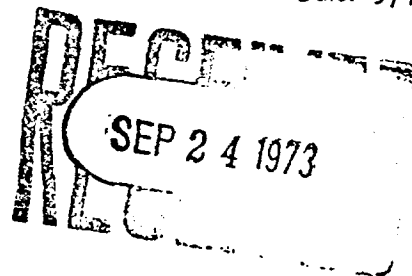
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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 9/7/73



1. Principal Investigator (give title and degrees):

Robert L. Volle, Ph.D., Professor
Edward G. Henderson, Ph.D., Associate Professor

2. Institution & address:

University of Connecticut
Health Center
Farmington, Connecticut 06032

3. Department(s) where research will be done or collaboration provided:

Pharmacology

4. Short title of study:

Molecular mechanisms underlying the development of tolerance to nicotine.

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 years

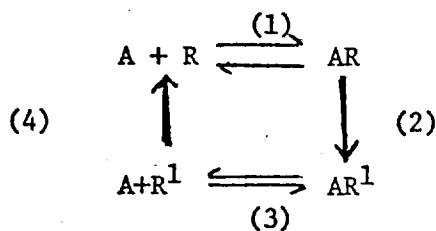
7. Brief description of specific research aims:

Our primary objective is to study the pharmacology of nicotine, lobeline and other nicotinic agents with the view of learning about central and peripheral toxicity to these drugs. Special emphasis will be placed on the study of the mechanism of tolerance to the nicotine drugs. It is well known that tolerance to nicotine develops when the compound is taken repeatedly. This is evident from the fact that confirmed tobacco smokers adapt to large amounts of the alkaloid while marked symptoms are exhibited by the tyro. A parallelism exists between this adaptive response to continued tobacco smoke and the adaptation or desensitization to nicotine which occurs at peripheral sites. A thorough examination of the process of desensitization at peripheral sites should be of great value in understanding central adaptive responses to nicotine. The proposed study will involve a further characterization of the mechanism of neuromuscular desensitization caused by lobeline and nicotine. The interaction of nicotinic agents with the neuromuscular junction and excitable tissues will be examined by radiochemical and electrophysiological techniques. It is expected that the proposed study will provide conclusive information as to the mechanism of lobeline's antagonistic actions toward nicotine and tobacco smoke and, in addition, will aid in understanding the underlying molecular mechanism involved in the desensitization process.

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8. Brief statement of working hypothesis:

According to the cyclic hypothesis of the mechanism of receptor desensitization (Katz and Thesleff, 1957):



The interaction of an agonist molecule (A) with the end-plate receptor (R, reaction 1) leads to a depolarization. Desensitization results from the gradual transformation of the receptor into a non-reactive form R^1 (reaction 2) which reverts slowly to R after the withdrawal of the drug (reactions 3 and 4). Desensitization is generally associated with the repolarization of the end-plate membrane; but receptor desensitization with lobeline and nicotine can occur, under appropriate conditions, without causing a depolarization (Hancock and Henderson, 1972; Steinberg and Volle, 1972; Volle and Reynolds, 1973). In fact, it has been suggested that the use of depolarization alone as a measure of the drug effect is unsatisfactory (Rang, 1971). Earlier studies have demonstrated that the E_m of end-plates of depolarized skeletal muscles was not changed by addition of acetylcholine (ACh) but that the electrical conductance of the post-synaptic membrane was raised (Castillo and Katz, 1955) and the inward and outward movements of sodium and potassium were increased (Jenkinson and Nicholls, 1961). From this evidence it is clear that the interaction of nicotinic agents with the post-synaptic membrane caused an alteration of ionic exchange without depolarization. Whether or not the modification of a particular ionic exchange mechanism by the nicotinic agent is involved in the desensitization process will constitute the major emphasis of this project.

9. Details of experimental design and procedures (append extra pages as necessary)

It is well known that nicotine has a bi-phasic action on neuromuscular transmission (Thesleff, 1960). Whereas small doses of nicotine facilitate the transmission of impulses across the neuromuscular junction, a blockade of transmission occurs when larger doses of nicotine are applied. This inhibition of transmission is also bi-phasic: the initial block (phase I) is associated with depolarization and the late block (phase II) is associated with repolarization (Hancock and Henderson, 1972). Nicotine is also capable of being transported across the muscle cell membrane, altering calcium movement, and possibly exerting a direct effect on the muscle membrane (Weiss, 1966a, 1966b).

In order for neuromuscular transmission to occur, a depolarization or reduction of the resting potential of the motor nerve terminal must take place which results in the release of acetylcholine (ACh). ACh then diffuses across the synaptic gap and exerts its action (depolarization of the skeletal muscle fiber) at a specialized postsynaptic site (end-plate) on the skeletal muscle fiber membrane. When the depolarization of the end-plate attains critical values, the generation of muscle action potentials occurs. The conduction of action potentials by the muscle fiber membrane is regenerative in nature and, like other electrically excitable tissues, is associated with specific changes in sodium and potassium permeability. Whereas excitability of the muscle cell membrane is associated with very specific changes in sodium and potassium permeability, depolarization of the end-plate is generally considered to be accompanied by nonspecific ionic permeability changes.

Accordingly, nicotine could exert its effects on muscle by causing the release of ACh from a presynaptic storage site, by combining with specific receptors of the end-plate, by altering the permeability properties of the muscle membrane, by activating directly the contractile mechanism or by a combination of these mechanisms. There is very little known as to the sites where nicotine exerts its influence and what specific fundamental physiological processes are involved.

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In contrast to nicotine, lobeline does not cause depolarization of the end-plate or the muscle fiber but it does cause an insurmountable blockade of the nicotine-induced depolarization (Steinberg and Volle, 1972; Hancock and Henderson, 1972; Volle and Reynolds, 1973). Nicotine reduces both quantal size and quantal content while lobeline reduces only quantal size, indicating that its effect is primarily post-synaptic.

Recent (unpublished) experiments in our laboratories indicate that lobeline (in the presence of d-tubocurarine) causes a frequency dependent depression of the twitch of directly stimulated frog sartorius muscle. Nicotine will also decrease the tension developed by directly stimulated muscle but the depression is transient and parallels the time course of the nicotine-induced depolarization-repolarization sequence. The nicotine induced twitch tension depression was blocked by d-tubocurarine while that caused by lobeline was not. This direct interaction of lobeline with the muscle cell membrane is suggestive of a local anesthetic type of interaction, since local anesthetics produce a similar response. Like lobeline, local anesthetics do not depolarize nerve or muscle fibers bathed in physiological solutions (Thesleff, 1956; Inoue and Frank, 1962) and block transmission at the myoneural junction (Thesleff, 1956; Castillo and Katz, 1957). The local anesthetic, procaine, has been demonstrated to interact with both pre- and postjunctional structures (Galindo, 1971), while only a postjunctional interaction of lobeline has been documented (Steinberg and Volle, 1972). In view of the similarities of the interactions of lobeline and local anesthetics with muscle cells and neuromuscular junctions, the fact that a prejunctional effect of lobeline has not been demonstrated is inconsistent. Therefore, it will be necessary to reexamine the effect of lobeline on neuromuscular transmission. The neuromuscular junction of both the frog sartorius and rat diaphragm will be impaled with microelectrodes and the parameters of transmitter release designated as quantal content (m) and quantal size (q) will be analyzed by measuring the variation of the amplitudes of end-plate potentials (EPP's) evoked by trains of stimulation of the sciatic nerve or phrenic nerve at rates of 5 to 20 impulses per second (Martin, 1966; Hubbard, *et al.*, 1969). In the previous study of this type it was found that the decrease in EPP amplitude caused by lobeline ($10^{-5}M$ to $2 \times 10^{-5}M$) was due to an interference with the postjunctional action of the transmitter as evidenced by a decrease in quantal size (q) without significantly altering quantal content (m) (Steinberg and Volle, 1972). In the proposed study concentrations of lobeline ($\geq 5 \times 10^{-5}M$) which produce a local anesthetic type block of muscle twitch will be employed. If the parallelism with local anesthetics persists, a decrease in both q and m would be expected.

In order to further evaluate the possibility of a local anesthetic type of interaction of lobeline with cell membranes, the effects of lobeline on the threshold for excitation of muscle cell membranes and membrane ionic conductance will be evaluated. It is characteristic of local anesthetic interaction with excitable tissues that the threshold for action potential generation is elevated in the presence of the local anesthetic. Therefore, the effects of lobeline on the directly stimulated muscle fiber will be evaluated and compared with typical local anesthetics. Methods to be employed will be similar to those previously described (Fatt and Katz, 1951; Castillo and Katz, 1955; Adrian, Constantin and Peachey, 1969). The end-plate and non-end-plate membrane will be impaled with two glass microelectrodes. One of the electrodes filled with $3M$ K^+ -citrate will be used to pass an electrical current while the other will be used to record the resulting voltage displacement. Both the current applied and the voltage displacement will be monitored with a Tektronix 502A oscilloscope and recorded with a Lehigh Valley Electronix moving film camera. The current-voltage records obtained will be analyzed in the manner originally described by Hodgkin and Rushton (1946). The data obtained in this manner can be used to calculate the total membrane conductance (G_m). The G_m reflects the total ionic exchange under the specific conditions to be employed.

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The depolarizing current necessary to cause a propagated action potential represents threshold for excitation. Current-voltage records and threshold stimulating currents will be measured before and after the application of lobeline, nicotine and local anesthetics. If lobeline modifies the measured parameters in the same way as typical local anesthetics, further evidence will be obtained to characterize the interaction of lobeline as local anesthetic-like.

An additional phase of this project will involve an examination of the mechanism responsible for the decreased rate of desensitization caused by depolarization of muscle cells (Magazanik and Vyskocil, 1970) and temperature reduction (Harris and Leach, 1968). It is suggested from these earlier studies that desensitization may involve the ionic channels of the membrane rather than or in addition to the receptors. Accordingly, a study will be made of the mechanism responsible for the decreased rate of desensitization to nicotine at low temperatures and in depolarized muscles. Both the reduced temperature and depolarization effects are suggestive of a Na^+/K^+ -ATPase inhibition. Since cardiac glycosides also inhibit the Na^+/K^+ -ATPase system, the effects of these agents on the nicotine-induced depolarization-repolarization sequence will be measured and compared with the effects of low temperature and K^+ -induced depolarization. The ability of lobeline to produce desensitization at low temperatures and in depolarized muscles will also be examined by the aforementioned electrophysiological methods.

In frog sartorius muscle there are more than one end-plate or chemosensitive sites per muscle fiber. While in most mammalian skeletal muscle fibers, the end-plate region of the neuromuscular junction usually occupies only a small fraction of the entire membrane surface. However, when the nerve supply to the skeletal muscle is severed and allowed to degenerate, extrajunctional receptors to ACh develop along the entire membrane surface (Thesleff, 1960; Miledi, 1960; Henderson and Hancock, 1971). The denervated muscle, then provides us with a system of expanded chemosensitivity, *i.e.*, a magnified end-plate, and provides a nearly ideal preparation for investigating the effects of nicotine and lobeline on the permeability of the end-plate and extrajunctional receptors to various inorganic ions.

Therefore, the normal frog sartorius and the denervated rat diaphragm muscle will be used to study the effects of nicotine and lobeline on end-plate phenomena. It has been demonstrated that it is possible to measure a significant increase in the rate of potassium (^{42}K) efflux induced by the application of nicotine to a normal frog sartorius muscle and that denervation does not intensify the response (Henderson and Hancock, 1971; Hancock and Henderson, 1972). In contrast, only the denervated mammalian muscle responds to nicotine with an increase in ^{42}K -efflux (Henderson and Hancock, 1971). The increased rate of ^{42}K -efflux in both preparations follows the same time course as the depolarization-repolarization sequence. Therefore, in normal physiological saline solutions it is not possible to dissociate the E_m change from the ionic flux changes.

Recently, it has been demonstrated that nicotine can produce receptor desensitization without depolarization by first applying the drug in either a sodium free medium or in a solution with elevated K^+ (Volle and Reynolds, 1973). The subsequent application of an equivalent or higher dose of nicotine in a physiological solution does not produce a depolarization equivalent to that which would be expected under normal conditions. It should be possible with this technique to measure the alterations of ionic exchange caused by nicotinic agents in the absence of depolarization.

A preliminary experiment demonstrating the effects of nicotine on the rate constant of ^{42}K -efflux from frog sartorius muscle is shown in fig. 1. Two muscles from a frog

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were incubated in radioactive (^{42}K) Ringer's solution overnight and then the washout or efflux of the radioactive tracer was followed in isotope-free, sodium-free (Tris- substituted for NaCl) by methods previously described (Sjodin and Henderson, 1964; Henderson and Volle, 1972). At the time indicated by the first arrow nicotine (0.05 mM) was added to the solution bathing one of the muscles (●) while carbachol (0.1 mM) was added to the solution bathing the other muscle. Both nicotinic agents caused a significant increase of the rate constant for ^{42}K -efflux. At the time indicated by the second arrow the drugs were removed and the rate of efflux returned to control levels. The third and fourth arrows indicate the second addition of equivalent concentrations of the drugs. These experiments indicate that the stimulation of ^{42}K -efflux by nicotinic agents can occur in the absence of depolarization and that this stimulation of K-exchange was not desensitized since the degree of stimulation produced by the second application of the drugs was equivalent to the first.

These experiments yield only preliminary findings and many more with varying doses of nicotinic agents and varying exposure times will be needed in order to evaluate the interaction of nicotine with the end-plate potassium ionophore. Similar experiments will also be undertaken with strips of denervated rat diaphragm muscle in order to compare the responses of the two species. Additional experiments employing agents which specifically block K^+ -exchange across the muscle cell membrane (e.g., 9-aminoacridine and Ba^{++} , Henderson and Volle, 1972) will be undertaken in order to determine whether or not the end-plate K^+ ionophore is the same as that of the surface membrane. Muscles will be bathed in a sodium-free solution containing one of the blocking agents and then the nicotinic agent will be added. If the stimulation of ^{42}K -efflux caused by nicotine is blocked by either 9-aminoacridine or Ba^{++} , it would be indicated that the end-plate K^+ channels are identical to those of the surface membrane.

Muscle cells depolarized by the elevation of extracellular K^+ concentration do not depolarize further after the addition of ACh but the end-plates respond to ACh with a change in electrical conductance and an increase in Na^+ and K^+ exchange (Castillo and Katz, 1955; Jenkinson and Nicholls, 1961). In addition, tetrodotoxin (TTX) and saxitoxin (STX) block conducted action potentials of nerve and muscle by specifically reducing the inward sodium current (Narahashi, Moore and Scott, 1964) while they do not prevent nicotinic agents from depolarizing the end-plate membranes (Elmqvist and Feldman, 1965; Kao and Nishiyama, 1965). Like the examination of K^+ -exchange, ^{22}Na -exchange will also be measured in this study under the aforementioned conditions. These experiments will allow us to determine whether or not the Na^+ -ionophore of the end-plate membrane is the same as that of the surface membrane and to determine the contribution of sodium to the nicotine-induced desensitization phenomenon. The specific method of measuring ^{22}Na -exchange will be with procedures previously described (Mullins and Frumento, 1963; Sjodin and Beauge, 1968; Henderson and Volle, 1972).

These radiochemical techniques will also be employed to further evaluate the possible local anesthetic response to lobeline, since it has recently been demonstrated that local anesthetics block ^{42}K -exchange in resting muscle cells (Henderson and Volle, 1973). The local anesthetics butacaine and procaine were shown to block ^{42}K -exchange without depolarization in Cl^- -containing media. In Cl^- -free media the blockade of ^{42}K -exchange by local anesthetics was identical but the local anesthetics produced a significant depolarization. Depolarization is an expected consequence of decreased K^+ -exchange and readily demonstrated when Cl^- conductance is diminished. Previous studies with lobeline involved measurements of E_m in Cl^- -containing media (Hancock and Henderson, 1972; Volle and Reynolds, 1973). In these experiments no depolarization was observed with lobeline concentrations up to $5 \times 10^{-5}\text{M}$. More recent preliminary experiments indicate that lobeline does cause a depolarization in Cl^- -free media. By analogy, it would be predicted that K^+ -exchange was depressed. The

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(ssibility of a lobeline-induced depression of K^+ -exchange will be examined both by electrophysiological and radiochemical techniques and compared with the local anesthetic induced blockade of K^+ -exchange.

In summary, this project will allow us to further evaluate the molecular mechanisms underlying the development of tolerance to nicotine and the antagonism between lobeline and nicotine. In addition, it is expected that the proposed study will allow us to determine whether a modification of the nicotinic receptor is involved in the desensitization process or an alteration of a particular ionophore. These studies of the interaction of nicotine and lobeline at peripheral sites will be of great value in understanding central adaptive responses to nicotine.

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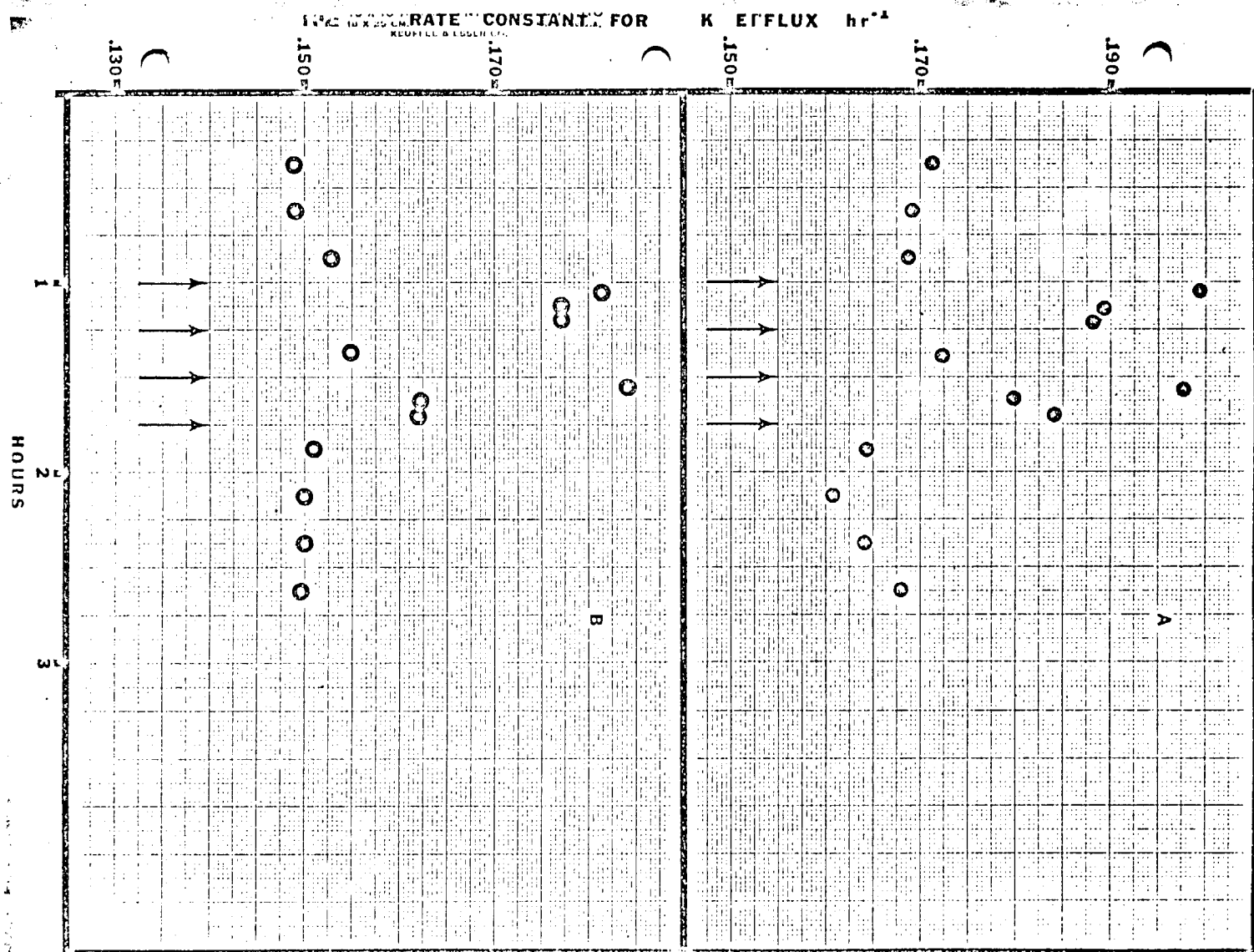
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Weiss, G. B. J. Pharmacol. Exp. Ther., 1966a, 154:595-604.

Weiss, G. B. J. Pharmacol. Exp. Ther., 1966b, 154:605-612.

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FIG. 1



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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The complete facilities of the Schools of Medicine and Dental Medicine will be available to the applicants. These include, The Health Center Library with a complete service for health related literature and a vivarium for quarantining the animal species to be used in this study. The applicants have at their disposal laboratory and office space containing equipment for radioactivity and ion analysis and equipment for electrophysiological recording.

Major items of equipment available to the applicants include: a dual channel Auto-gamma spectrometer, three channel liquid scintillation spectrometer, atomic absorption spectrophotometer, flame photometric instrumentation, freezing point depression osmometer, desk top computer, dual channel oscilloscopes, square pulse generators, moving film recording camera, microelectrode puller and various high input impedance, capacity neutralization amplifiers. In addition, the facilities of the pharmacology department will also be available to the applicant. These include: a complete dark room, cold room, warm room and dishwasher-sterilization room.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

R. L. Volle, Ph.D.

25

-

E. G. Henderson, Ph.D.

50

-

Technical

Research Assistant III Fringe Benefits (29%)
(To be appointed) (\$2,755.)

100

12,255.

Sub-Total for A

12,255.

B. Consumable supplies (by major categories)

Radioactive isotopes

3,000.

Animals

1,000.

Chemicals, glassware, etc.

800.

Electrophysiological and photographic supplies

500.

Sub-Total for B

5,300.

C. Other expenses (itemize)

Principal investigator's travel for the presentation
of findings at scientific meetings (Pharmacol. Soc.
and FASEB)

400.

Maintenance of equipment

800.

Page charges and reprints of publications

400.

Sub-Total for C

1,600

Running Total of A + B + C

19,155.

D. Permanent equipment (itemize)

None

Sub-Total for D

-

E

2,873.

Total request

22,028.

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|---------|
| Year 2 | 13,235. | 5,000. | 1,600. | - | 2,975. | 22,810. |
| Year 3 | 14,294 | 5,000. | 1,600. | - | 3,134. | 24,028. |

List financial support from all sources, including own institution, for this and related research projects.

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|--|---------|--------------------|
| Studies of Sympathetic Ganglia Following Conditioning (Volle) | PHS Grant 5R01-NS07540-07 | 21,806. | 9/1/73 - 8/31/74 |
| Nicotinic Receptors in Muscle Membrane: A Study of Receptor Desensitization (Volle) | AMA-ERF (Terminal Year) | 15,792. | 8/1/73 - 7/31/74 |
| The Pharmacology of Glycerol- treated Striated Muscle (Henderson) | University of Connecticut Research Foundation | 2,900. | 5/15/73 - 5/15/74 |

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--------------------------------|---------|--------------------|
| Pharmacological Modification of Ionic Exchange (Henderson) | AMHL 17386-01 | 29,515. | 1/1/74 - 12/31/74 |

it is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Robert L. Volle
Signature Robert L. Volle Date 9/7/73
Telephone 203 674-2120 -
Area Code Number Extension

Checks payable to

James C. Leming, Assistant Vice-President
For Financial Affairs

Mailing address for checks

University of Connecticut Health Center
Farmington, Connecticut 06032

Responsible officer of institution

Glenn W. Ferguson, President or
Typed Name Edward V. Gant, Provost

Title President or Provost

Signature Ethan V. X. [illegible] Date 9/18/23
Telephone 203 486-2338 or 203 486-2418
Area Code Number Extension

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#960 - GIRI

1003540376

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

February 7, 1974

Grant application No. 960

MISCELLANEOUS

To: The committee comprising Drs. Bing, Huebner and Meier

Subject: Shri N. Giri, Univ. of Calif. School of Veterinary Medicine, Davis
New application No. 960
"Cigarette Smoke and its Effects, in Man and Animals, on the
Metabolism of the Well-Known Pesticide DDT and DDT Metabolites"

History

This proposal was initially handled as case No. 250 in the name of
Mr. Richard K. Lee, a graduate student. Application was encouraged.

Request

Application No. 960 requests \$23,865 plus one additional year.

Document Submitted (attached)

1. Application dated 1/18/74 (5 pages plus inserts).
2. Biographical sketches of Mr. Lee and of Drs. Giri and Peoples.

FWN:gh

Enclosures

FWN
F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

JAN 30 1974

Date: 1/18/74

1. Principal Investigator (give title and degrees):
 - a) Richard K. Lee, Graduate Student, M.S.
 - b) Shri N. Giri, Assistant Professor of Pharmacology, Ph.D.,
Faculty Principal Investigator.
 - c) Stuart A. Peoples, Professor of Pharmacology, M.D., Faculty Principal Investigator.
2. Institution & address:
School of Veterinary Medicine
University of California
Davis, California
95616
3. Department(s) where research will be done or collaboration provided:
Department of Physiological Sciences
4. Short title of study:
Cigarette Smoke and its Effects, in Man and Animals, on the Metabolism of
the Well Known Pesticide DDT and DDT Metabolites.
5. Proposed starting date: July 1, 1974
6. Estimated time to complete: One year
7. Brief description of specific research aims:

To determine whether cigarette smoking in humans results in lower endogenous levels of DDT and its metabolites, and concurrently to determine whether cigarette smoking enhances the rate of metabolism of exogenous DDT which may enter by the diet and or by intentional oral administration. In both cases to also determine whether a concomitant excretion as DDA, the principal urinary metabolite of DDT, is observed.

To determine the mechanisms involved for the proposed increase in DDT metabolism as a result of exposure to cigarette smoke.

To determine the constituent of tobacco smoke which is responsible for the postulated increase in DDT metabolism in those who smoke. Whether the causative agent of tobacco smoke is nicotine, benzpyrene or some other component of the tobacco smoke.

To determine the feasibility of using Urinary DDA levels to measure the microsomal enzyme induction state of man and animals without altering the homeostatic balance of the experimental subject.

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This author has previously made a finding that there are in fact higher levels of Urinary DDA in those who smoke. It is believed that such an increased level of DDA is a result of an enhanced ability, by those who smoke, to metabolize both exogenous and endogenous DDT and or metabolites of DDT. The result of such an increase in metabolism being the beneficial ability to excrete DDT and or its metabolites, from the body, as Urinary DDA. For this reason, it is believed that a study of the effects of cigarette smoke on the levels of DDT, its stored metabolites and its urinary metabolite, DDA, is indicated.

It is also believed, due to the ubiquitous nature of DDT, that the use of Urinary DDA as a tool for measuring the microsomal enzyme induction state of man and animals should be investigated.

9. Details of experimental design and procedures (append extra pages as necessary)

Experiment 1: Procedure for the determination of the effects of cigarette smoking on the metabolism of DDT and metabolites in man.

Approximately fifty cigarette smokers, of which half will be male and half will be female, will be obtained. Classification will be according to the number of cigarettes smoked per day as well as to the length of time they have been smoking. An equal number of controls will also be selected.

Blood samples and twenty four hour urine samples will be taken. A correlation between blood levels of DDT, DDE and possibly DDD with the urinary levels of DDA will be made and any distinction between smokers and nonsmokers will be noted.

Blood analysis for DDT and metabolites will be by the modified Dale Method (Nachman et al., Health Lab Sci. 6: 148, 1969). Urinary DDA analysis will be determined by the method which made the initial finding, concerning the relationship between cigarette smoking and Urinary DDA levels, possible (Lee et al., Proc. West. Pharmacol. Soc. 16: 240, 1973).

Experiment 2: Procedure for determining the mechanisms involved which account for the observed increased levels of DDA in the urine of those who smoke.

Experiment 2-A: In vivo effects of cigarette smoke on the metabolism of DDT and DDE in rats.

A lighted cigarette will be attached to one end of a six liter chamber made to house four rats. Air will be drawn through the chamber. The rats will be exposed to five cigarettes per hour for four hour intervals each day for a total of two days.

Twenty four hours after the last exposure, 30 mg/kg p,p' DDT will be administered to one group of rats i.p. and 30 mg/kg p,p' DDE will be administered i.p. to another group. An equal volume of solvent will be administered to a control group. Urine will be collected at twenty four hour intervals for two

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2a

days. DDA analysis will be as previously described and a determination of whether cigarette smoke increases the metabolism of either DDT and or DDE should be possible.

Experiment 2-B: In vivo determinations in rats of the effects of cigarette smoke on the metabolism of DDT-¹⁴C and DDE-¹⁴C.

Rats will be exposed to cigarette smoke as previously described. DDT-¹⁴C will be administered i.p. to one group and DDE-¹⁴C to another. Dosage for both isotopes will be 4 mg/kg, Sp. A. 1 mc/mM. Controls will be given an equal volume of the solvent. Urine will be collected for a twenty four hour period and at the end of said period, a venous blood and fat biopsy sample will be taken. Determination of the metabolites will be by thin layer chromatography and subsequent quantitation by scintillation counting (Datta et al., Tox. Appli. Pharm. 6: 321, 1964).

Experiment 2-C: In vitro effects of cigarette smoke on the metabolism of DDT and DDE.

Again, rats will be exposed to cigarette smoke and lung and liver homeogenates will be made (Alary et al., Tox. Appli. Pharm. 18: 457, 1971). Subsequently, p,p' DDE and p,p' DDT will be added to different homeogenates (0.1 ml of 1 mg/ml) and incubated. Analysis for parent compounds and metabolites will be made (Saschenbrecker et al., J. Agr. Food Chem. 15: 168, 1967). A possible inquiry into the use of kidney tissue may be made.

Experiment 3: Procedure for determining the constituent of cigarette smoke which is responsible for the observed increase in Urinary DDA in those who smoke.

The use of known microsomal enzyme inducers, which are known to be present in tobacco smoke, will be utilized in an attempt to elucidate the mechanism involved for the observed increase in Urinary DDA levels in those who smoke.

Initial experiments will be conducted through the use of nicotine and 3, 4-benzpyrene. Rats will have their microsomal enzymes induced by these compounds and experiments analogous, and in a similar step wise manner, to those previously described will be performed.

Rats will be given nicotine sulfate, 2 mg/kg/2 ml s.c. daily for fourteen days. Controls will receive saline, 2 ml, 0.85 g/ml w/v NaCl/kg. Another group of rats will be given 3, 4-benzpyrene, 0.5 ml of 20 mg/kg in corn oil, for one day.

After induction of the microsomal enzymes the following experiments, which have been previously described in more detail, will be attempted.

Experiment 3-A:

Rats whose microsomal enzymes have been induced by either nicotine or benzpyrene will be administered a large i.p. dose of either p,p' DDT or p,p' DDE (30 mg/kg). The urine will be collected for twenty four intervals and analyzed for the presence of DDA.

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Experiment 3-B:

Rats will be administered nicotine and benzpyrene as described. This will be followed by an i.p. administration of DDT- ^{14}C or DDE- ^{14}C , 4 mg/kg, Sp. A. 1 mc/mM. A correlation between DDT and its metabolites in blood, fat and urine by thin layer chromatography and scintillation will be made. A similar analysis will be made for DDE.

Experiment 3-C:

After inducing the microsomal enzymes of the rats with either nicotine or benzpyrene, lung and liver homogenates will be made. Subsequently p,p' DDT and or p,p' DDE (0.1 ml of 1 mg/ml) will be added to the separate homogenates and incubated. Analysis for parent compounds and metabolites will be made. A possible inquiry into the use of kidney tissue may be made.

Experiment 4: Procedure for the determination of whether Urinary DDA levels can be used as a means of measuring the induction state of the microsomal enzymes of man and animals.

Experiment 4-A: The use of humans who are receiving treatment for epilepsy with either phenobarbital or diphenylhydantoin, both being known microsomal enzyme inducers, will be made.

Urine will be collected from out patients in the Epilepsy Clinic, Sacramento Medical Center, Sacramento, California. A correlation between Urinary DDA levels and the administration of phenobarbital and or diphenylhydantoin will be made.

Experiment 4-B: Microsomal enzyme induction in rats and its effects on the Urinary DDA levels.

Rats will be used as the experimental subject placed five per metabolism cage from which their urine will be collected at three hour intervals. Three groups of five rats will be used as controls. Experimentals will be administered pretreatment i.p. doses of phenobarbital each day (75 mg/kg) for one week. Controls will be administered a like volume of saline. Diet will be ad libitum and will be measured. Urine will be collected and analyzed for DDA. Hopefully, a difference in metabolism of dietary DDT will be observed as the enzymes are induced. At random, hexobarbital (125 mg/kg) will be injected into a control and experimental to measure the sleeping time and to delineate whether induction of microsomal enzymes is actually taking place.

At the end of the pretreatment period an i.p. dose of DDT (30 mg/kg) in corn oil will be given to all animals to determine whether there is in fact an increased metabolism of DDT to DDA. A sufficiently large dose of DDT is utilized to eliminate any contribution from endogenous DDT and or metabolites.

Of major significance of this part of the proposal is the possible use of Urinary DDA as a tool for measuring the induction state of the microsomal enzymes of humans. This is of particular importance for patients receiving known microsomal enzyme inducers, or a combination of such inducers.

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That one could test the microsomal enzyme induction state of man or animal, as is; that is without altering the homeostatic balance of the subject (due to the ubiquitous nature of DDT), would seem to be significant contribution of this proposal. This is emphasized when one considers the ease with which Urinary DDA samples can be obtained and the facility with which they can be analyzed (Lee et al., Proc. West. Pharmacol. Soc. 16: 240, 1973).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

1. Aerograph 1520 Gas Liquid Chromatograph.
2. Walton Horizontal Smoke Exposure Machine.
3. Beckman LS- 200 B Liquid Scintillation Spectrometer.
4. Isco Fraction Collector.
5. Sorvall Model RC 2-B High Speed Refrigerated Centrifuge.
6. Cold room.
7. Warburg Apparatus.
8. Virtis Extracto-matic.
9. Space available includes research laboratories, office space and animal housing.
10. Mettler H-15 Analytical Balance.

11. Additional facilities required:

None required.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

Shri N. Giri, Ph. D., Assistant Professor

% time

Amount

11

\$2,975 (including 15% fringe benefits)

Shri N. Giri, Ph. D., Assistant Professor

4

--0--

Stuart A. Peoples, M.D., Professor

5

\$1,970 (including 15% fringe benefits)

Stuart A. Peoples, M.D., Professor

2

--0--

*In keeping with policy of the Veterinary Medical Strict Full-time salary plan, 75% of faculty effort associated with the project must be charged to the project.

Technical

Richard K. Lee, M.S., Staff Research Associate, Step II 100

\$11,758 (including 12% fringe Benefits)

Sub-Total for A \$16,703

B. Consumable supplies (by major categories)

Animals (300 CRD Free Rats)

\$1200

Animal Care (Rats; 6¢/day)

150

DDT-¹⁴C, Ring Labeled, 1.0 mCi.

\$1500

Chemicals

100

Regular Lab Supplies

100

Sub-Total for B \$3050

C. Other Expenses (itemize)

Principal Investigators to attend meetings

500

Maintenance and repair charges for existing equipment

500

Sub-Total for C \$1000

Running Total of A+B+C \$20,753

D. Permanent equipment (itemize)

Sub-Total for D --0--

E. Indirect costs (15% of A+B+C)

E \$3,112

15. Estimated future requirements:

Total Request \$23,865

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|--------|----------|-------------------|----------------|------------------|----------------|----------|
| Year 2 | \$16,703 | \$3040 | \$1000 | --0-- | \$2823 | \$23,865 |

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|--|----------|--------------------|
| 1. Physiological Basis For the Genesis of Pulmonary Edema. | Tuberculosis and Respira- tory Disease Association of California | \$ 7570 | 7/1/73 to 6/30/74 |
| 2. Studies on the Avicide 1339. | Starling Research | \$10,000 | 7/1/73 to 6/30/74 |
| 3. Forensic Toxicology | Robert L. King, Associates | \$ 1000 | 7/1/73 to 6/30/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|--|----------------------------------|-----------|--------------------|
| 1. Role of Lung Microsomal Enzymes in Pathogenesis of Chemically Induced Pulmonary Edema and Fibrosis | American Lung Association | \$19,679 | 7/1/74 to 6/30/75 |
| 2. Edema formation and Drug Metabolism in the Lung. | National Institutes of Health | \$219,345 | 5/1/74 to 4/30/77 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

The Regents of the University of
California
Mailing address for checks
Davis, California 95616

Principal investigator

Typed Name Shri N. Giri, Ph.D.Signature Shri N. Giri Date 21st Jan. 1974Telephone (916) 752-1598

Area Code Number Extension

Responsible officer of institution

Typed Name Allen G. MarrTitle Dean, Research DevelopmentSignature Allen G. Marr Date 1-25-74Telephone (916) 752-0650

Area Code Number Extension

1003540385

#912-McCLUGAGE

1003540386

Drs. I. I. I.
Jacobson
Meier

Pharmacology
Miscellaneous

#912

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

MAY 29 1973

Date: May 16, 1973

1. Name of Investigator(s): (include Title and Degrees)

Samuel G. McClugage, Jr., Ph.D., Assistant Professor, Department of Anatomy
Marilyn L. Zimny, Ph.D., Professor, Department of Anatomy

2. Institution &

Address: Louisiana State University Medical Center
Department of Anatomy
1100 Florida Avenue
New Orleans, Louisiana 70119

3. Short Title of Project:

Microvascular Response of Fetus to Carbon Monoxide or Nicotine

4. Proposed Starting Date:

October 1, 1973

5. Anticipated Duration of this Specific Study:

October 1973 through September 1976

Brief Description of Objectives or Specific Aims:

(PLEASE SEE PAGE 2)

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7. Give a Brief Statement of your Working Hypothesis:

(SEE PAGE 3)

6. Brief Description of Objectives or Specific Aims:

There are increasing numbers of reports in the literature which suggest that smoking during pregnancy can cause alterations in the normal development of the fetus in utero. Some of the alterations described are decreased neonatal birth weights, greater incidence of premature delivery, increased incidence of spontaneous abortion, and a higher incidence of stillbirths or neonatal deaths of children born from mothers who smoke. Other authors disagree with the causal relationship between impairment of fetal development and maternal cigarette smoking since they believe that social class, background, and other environmental factors which may affect the mother can have just as profound an effect on the fetus as maternal smoking per se. In the past, many have felt that the nicotine content of cigarette smoke may be the etiologic agent causing alterations in the fetus by possibly crossing the placental barrier. Since nicotine has been demonstrated to have so many pharmacological effects on animals and even man, it was only natural to strongly suspect that it was the harmful agent in cigarette smoke. However, when one examines a list of compounds which have been isolated in the gaseous phase of cigarette smoke, he can readily identify other substances which may too have an effect on a growing fetus in utero. One such compound is carbon monoxide (CO). Cigarette smoke is known to have a relatively high content of CO which in living animals competes with oxygen for binding by hemoglobin. This binding of CO by hemoglobin forms an inactive pigment called carboxyhemoglobin (COHb) which causes a proportional decrease of the oxygen carrying capacity of the blood by shifting the oxyhemoglobin dissociation curve to the left (decreases the unloading tension of oxygen). Since CO is known to cross the placental barrier in various

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animals and man, it may be responsible for the reported effects of maternal smoking on fetal development which heretofore may have been attributed to nicotine. Thus, using an in vivo microscopic method, a study will be conducted in rabbits to observe the responses of the fetal microvascular system after the maternal exposure of carbon monoxide in one group and after the exogenous administration of nicotine to the mother in a second group. The fetal microvascular response in these two experimental groups will then be compared to that of the mother. Exactly how the maternal exposure to cigarette smoke containing the CO and nicotine causes the reported alterations in the fetus has not been adequately described due, in part, to the difficulty in studying in vivo the fetus while maintaining homeostasis. After in vivo observations, tissue samples from the microvessels will be taken in order to prepare them for transmission or scanning electron microscopy. Thus, the in vivo observations can be correlated with tissue sections selected for study by scanning or transmission electron microscopy. This study is designed to specifically examine in vivo the separate effects of nicotine or CO on the fetal microvascular system in an attempt to provide further information on the reported harmful effects of cigarette smoke on the unborn and even adults; thus, the adverse effects of maternal smoking on fetal development and its reported etiologic role in the development of cardiovascular diseases in adults may be better understood.

7. Brief Statement of Working Hypothesis:

Due to previous work done in my laboratory and work done by others, CO may induce alterations in the fetal or adult microvascular system which seriously compromise blood flow to tissues or organs. This reduction of blood flow would seriously reduce the oxygenation of fetal or adult tissues; this, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of

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COHb. Nicotine has not yet been studied in this regard. In vivo microscopy offers the possibility of measuring vascular dimensions and at the same time observing any changes in the behavior of blood in the microvessels. It is quite conceivable that carbon monoxide and/or nicotine may both alter the dynamic structure and function of the fetal or adult microvascular system which, in turn, will reduce the proper delivery of blood to tissues or organs.

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8. Experimental Design and Significance:

A. Experimental Design:

In one experimental group, the mesenteries of fetal and adult pregnant rabbits (New Zealand albino) will be studied. The pregnant rabbits will be anesthetized with Urethane I.V. (ethyl carbamate) or with methoxyflurane using a closed circuit anesthetic machine. To study the fetal circulation, a fetus is exteriorized from the uterus of the mother leaving the placental circulation and fetal membranes intact on various days of gestation between days 25 and 32 and the fetal mesentery is exposed surgically. Homeostasis is maintained by irrigating the field of study with Ringer's solution warmed to the body temperature of the animal by regulating heaters. Gauze sponges covering the fetus and surrounding the mesentery provide insulation for the animal during the experiment. Furthermore, ambient air surrounding the fetus is maintained at bodily temperature (37.5°C) using a Sage air incubator. To study the mesentery of the adult animal, the bowel is displaced after laparotomy and a loop of bowel is exposed. Homeostasis is maintained as in the fetus.

To study the exposed mesentery of the fetus or adult pregnant animal, a beam of monochromatic or white light is brought to the undersurface of the mesentery via a hollow, fused quartz rod; subsequently, the mesentery is transilluminated and examined with a Leitz stereo binocular microscope equipped with 2.5X, 4X, and 10X objectives and 10X and 16X oculars. Measurements of the microvasculature within the mesentery will be performed by a Leitz eyepiece micrometer. Alternatively, the optical images from the microscope will be projected onto the photocathode of a Cohu, RCA, or Fairchild/Dumont vidicon television system and kinerécored with a Bolex H-16 Rex 5 16mm. motion picture

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camera. The use of monochromatic light used in conjunction with a black and white television system greatly improves the visualization of living tissues or organs since the contrast is greatly increased. The 16 mm. motion picture camera may also be used for direct cinéphotomicrography. Throughout the in vivo experiments, the results are permanently documented for later reference. These results can be studied repeatedly and critically analyzed frame by frame in order to compare the sequential responses of the microvasculature in one animal to that of other animals. Thus, using this in vivo technique, the rate, duration, magnitude and direction of the response in the fetus or adult animal can be examined and recorded.

In one group of experimental animals, studies will be continued on the response of the fetal microvascular system to the maternal exposure of CO. To study the acute response of the fetal microvasculature to CO, the mother will receive a mixture of CO and air in varying concentrations of CO from .01% to .1% balance/air (100-1000 p.p.m.) using a closed circuit anesthetic machine. This range of CO will cause an increase in the COHb level of the female adult rabbit from 5 - 15% which mimicks COHb levels which have been reported in human studies on mothers who smoke. The fetal microcirculation will then be studied under the following experimental conditions: (1) after maternal anesthesia but before CO exposure, i.e., while the mother is breathing room air or air via the closed circuit anesthetic machine; (2) during maternal CO exposure; (3) during recovery when the mother is again allowed to breath room air. It should be emphasized that each pregnant animal can be used for each of the above experimental (CO) groups. Thus, each animal can be used as its own "control". The response of the fetal microvascular

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system to CO will be compared to that of the adult microvascular system in order to compare the sensitivity of the fetus with that of the mother.

In a second group of experimental animals, the effects of nicotine on the fetal microcirculation will be examined after the administration of a subcutaneous dose to the mother. The dose to be used in rabbits will attempt to mimic that amount of nicotine absorbed by human smokers, which has been reported to be 1.0 to 2.0 mg. of nicotine per kilogram from a pack of cigarettes per day. As in the CO experimental group, each fetal preparation will be examined before, during, and after the maternal administration of nicotine. The response in the fetus will also be compared to that of the adult microvascular system.

After completion of experiments on the effects of CO or nicotine on the fetal microvascular system, the results from the two experiments will be compared for any similarities or differences in responses under the two experimental conditions. A third group of experiments which would be most interesting to perform would be to study the response of the fetal microvascular system during the maternal inhalation of cigarette smoke. However, to date, I am not aware of any mechanical device for exposing rabbits to cigarette smoke under conditions which simulate human exposure. There are, of course, means by which the effects of cigarette smoke could be examined in rabbits, but I question the value of these studies if they do not at least simulate conditions during human smoking. If such a device for rabbits becomes available during the course of the experiments, it could be easily incorporated into the experimental design of this study. I know that the Council for Tobacco Research is sponsoring work to develop mechanical devices for animals which simulate human conditions; thus, they are in a position to know when such a device for use with rabbits becomes available. The in vivo model which is to be used in the

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various experimental groups would be a means for examining the effects of maternal smoking on the response of the fetal microvascular system, and then to compare this response with the nicotine-treated and CO-treated animals.

One further experimental group of animals will be used to study in adult female Sprague-Dawley rats (100-125g) the response of the mesenteric and hepatic microvascular system to carbon monoxide or nicotine. Animals will be anesthetized by intraperitoneal injection of urethane (ethyl carbamate). To expose the liver or mesentery of the rat, a midline and subcostal incision will be made and the liver or mesentery exteriorized by floating it onto a window of Saran Wrap which overlays a substage condenser of a Leitz Panphot microscope. Homeostasis will be maintained by irrigating the field with physiological Ringer's solution kept at the body temperature of the animal by heat regulators. Transillumination of the tissue will be accomplished by using a technique modified after Bloch and Coyas (Anat. Rec. 145: 374, 1963). With the liver or mesentery in position over the substage condenser, microscopy of the tissue is accomplished by passing a beam of monochromatic light through the condenser of a modified Leitz Panphot microscope. As mentioned earlier, the use of monochromatic light aids in the visualization of tissues and when used in conjunction with a black and white television system, the contrast can be greatly improved. The transilluminated tissues can then be observed by direct microscopy at magnifications of 100-1200X using Leitz water immersion or U.M.K. objectives with appropriate oculars or the optical image can be projected onto the photocathode of a television system.

The adult rats will be exposed to CO and nicotine in a similar manner as described for rabbits before, during, and after exposure. This experimental

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group of animals will provide information on another species of animal which can then be compared to the response of the microvascular system in adult rabbits. Furthermore, the use of a microscope (Leitz Panphot) which permits higher magnifications (100-1200X) than a stereo-binocular microscope may provide information which would not be obtainable with lower magnifications and poorer resolutions. Also, the microvascular system of liver is morphologically and functionally different from that of the mesentery; thus, the sensitivity of the two to CO or nicotine may also be quite different since one represents a microvascular bed in a relatively non-metabolic tissue (mesentery) versus one which is highly metabolic (liver).

During the experiments, the maternal and fetal hematocrit, hemoglobin concentration (g/100ml), oxyhemoglobin concentration (%) and carboxyhemoglobin (%) will be monitored, the latter three by an IL CO-Oximeter, in order to compare the response in the fetal or adult microvascular systems with any fluctuations in maternal or fetal blood parameters.

In each of the various experimental groups, samples of blood vessels will be taken after in vivo observations. These samples of blood vessels will be fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4 for 24 hours, rinsed three times with buffer and stored in a refrigerator. Part of the sample will then be osmicated, dehydrated in graded alcohols, processed through amyl acetate and critical point dried. After drying, the tissue will be coated with carbon and gold palladium alloy and viewed in a JSM-U3 scanning electron microscope. The usual accelerating voltage used by the investigator in past studies of other tissues has been 15 KV. Observations of the tissue in question will be made at this accelerating voltage and other magnitudes of voltage will be tested so as to obtain the maximum visual results.

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Following scanning electron microscopic observations, the sample may be placed in propylene oxide and embedded in plastic for further study with the transmission electron microscope. The plastic embedment material used in our laboratory is either Maraglas or Araldite. After polymerization of the plastic embedment, 1 μ plastic sections will be stained with Paragon and viewed with a light microscope for purposes of orientation. Ultra thin sections will then be made, stained with uranyl acetate and lead citrate and viewed with an A.E.I.-6B transmission electron microscope.

In addition to viewing the same piece of tissue with both scanning and transmission electron microscopy, it will also be possible to use part of the original sample that was fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4, solely for transmission electron microscopic observation. For this, part of the original fixed sample would be osmicated, dehydrated in graded alcohols, placed in propylene oxide and subsequently be embedded in Maraglas. Once again thick plastic sections would be stained with Paragon and the following ultra thin sections would be stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope.

If deemed necessary, for correlation with scanning or transmission electron microscopic observations, part of the originally fixed sample can also be prepared for light microscopy. For this purpose part of the original fixed sample would be dehydrated in graded alcohols and embedded in paraffin. Paraffin sections could then be stained for routine histological observations or stained with special chemicals so as to visualize various fibrous components of the tissue or possible lipid inclusions.

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B. Significance:

The specific response of the fetal microvascular system to maternal exposure of carbon monoxide or nicotine has not been reported due, in part, to the difficulty in studying these vessels in vivo with the light microscope while maintaining homeostasis. In general, the response of the fetal microvascular system to any maternal agent is poorly understood due, in part, to the lack of information in man and animals regarding the transfer of substances across the placenta to the fetus.

During this past year, my laboratory has been conducting in vivo studies on the response of the fetal microvascular system to maternal carbon monoxide exposure. The main purpose of this study was to observe any changes in the dynamic structure or function of the fetal microvascular system which may occur after exposure of the mother to carbon monoxide in order to possibly explain the reported cause-and-effect relationship between maternal smoking and impairment of fetal growth and development. Since mothers who smoke have increased circulating carboxyhemoglobin (COHb) levels, possibly the carbon monoxide (CO) per se may have detrimental effects upon the fetus, particularly since CO is known to cross the placental barrier.¹ In this regard, Astrup et al.² found that mothers exposed to 180 p.p.m. of CO had a 20% decrease in birth weight and a neonatal mortality rate of 35%. They suggested that the CO content of cigarette smoke may be responsible for these two occurrences. In studies conducted by Meyer and Comstock,³ perinatal mortality increased if the mother had smoked during pregnancy. Several authors⁴⁻⁹ have suggested that the lower birth weights and increased mortality of babies born from mothers who smoke may be related to the relative hypoxemia in the fetus caused by the CO since babies born at high altitudes often have similar

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incidences of lower birth weights or mortalities. Many of these reports strongly suggest a cause-and-effect relationship between CO and fetal development in mothers who smoke. The hypoxemia which occurs from exposure to CO is most often mentioned as the harmful effect elicited by CO per se, either from cigarette smoke or other sources. If the hypoxemia is truly responsible for the pre-natal or post-natal alterations from mothers exposed to CO, then the only way this hypoxic effect might be overcome would be by either increased production of hemoglobin by the mother, or an increased maternal blood flow to the placenta.

Experiments conducted in my laboratory during the past year, however, suggest that CO may have other effects upon the fetus in addition to its known effects upon the oxyhemoglobin dissociation curve. My experiments to date have shown that carbon monoxide administered to the mother at concentrations of 100-1000 p.p.m. in an air mixture will cause an increase in the maternal and fetal COHb level in rabbits comparable to or slightly higher than that of smokers (5-25% COHb). The exposure to CO in the mother causes a linear increase in her COHb levels throughout a four hour observation period. Fetal blood samples taken after completion of in vivo observations on the fetal mesentery revealed a similar or slightly higher per cent of saturation of hemoglobin by carbon monoxide. The oxyhemoglobin level (%) decreased in the adult concomitant with the rise in COHb levels. The hematocrit and total hemoglobin (g/100ml) did not change appreciably throughout the course of the experiments.

The response of the fetal microvascular system to increased levels of COHb is a vasoconstriction in the small arteries and veins (100-300 μ I.D.) of the mesentery followed by a progressive decrease in the linear velocity of blood flow through these vessels. These hemodynamic events preceded the eventual breakdown of the endothelial lining of the capillaries and

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post-capillary venules resulting in the formation of petichial hemorrhages along the course of these vessels (capillaries and post-capillary venules) and widespread congestion within the capillary network of the fetus. The per cent of vasoconstriction (compared with control, before CO administration) in the fetus increased with time and with the level of maternal COHb. The observation period was never longer than four hours. Furthermore, the degree of extravasation of red blood cells from the capillaries or post-capillary venules and the amount of congestion within the microscopic field also increased with time and with the level of maternal COHb. The maximal response to the increased COHb levels was cessation of flow through terminal arterioles, capillaries, and post-capillary venules, with a great reduction in the linear velocity of blood flowing through the small arteries and veins in the mesentery. Due to the congestion within the capillary bed, the majority of the blood flowing in the small arteries would bypass the capillary network by flowing into arteriovenous anastomoses into small veins or venules. Control animals allowed to breath room air or an air/gas mixture did not develop the microvascular lesions observed in fetuses exposed to CO for similar periods of time up to four hours.

Once a high level of maternal COHb was reached, the toxic effects of CO on the fetus were not reversible since removal of the CO stimulus after the initial vasoconstriction does not reverse the further effects upon endothelial permeability of capillaries and post-capillary venules. This is probably explained by the fact that the COHb levels, once elevated (20-25%), will not fall in time to prevent further damage to the endothelium of the capillaries and post-capillary venules. The results suggest, however, that if the CO stimulus is removed before the COHb levels reach 10% in the mother, only a slight vasoconstriction of small arteries and veins will be observed.

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It is interesting to compare the results of these experiments with those experiments performed in the past on the response of the fetal mesenteric microvascular system to maternal hypoxia¹⁰. In this earlier study, maternal hypoxia induced a vasoconstriction in the fetal microvascular bed which seemed to be mediated by an oxygen dependent alpha-adrenergic mechanism since recovery from this vasoconstriction coincided with the return of the pO_2 to normal values after removal of the hypoxic stimulus to the mother. In these experiments, this recovery occurred within 20 minutes.

The results of my experiments suggest that the vasoconstriction in the fetal microvascular system may be due to fetal hypoxemia which occurs with increased levels of COHb since hypoxia alone, induced by a low oxygen gas mixture, produced a similar vasoconstriction. Other authors¹¹ have also reported increased fetal systemic vascular resistance during hypoxia in pregnant ewes, although one cannot assume that an increased fetal systemic vascular resistance reflects what is occurring in a particular microvascular bed of an animal.

It is more difficult to explain the endothelial damage induced by CO per se or hypoxia. In this regard, the extravasation of red blood cells through the endothelium represents some type of endothelial damage. This increased permeability of endothelium after exposure to CO has also been described by other authors in "adult" animals or human studies. Astrup¹² found that cholesterol-fed adult rabbits exposed to CO had a greater accumulation of cholesterol in their arterial walls (aorta) when compared to only cholesterol-fed controls. Furthermore, Astrup and his associates found that CO (9-10% COHb) alone induces arterial lesions hallmarked by subendothelial edema indistinguishable from the intimal appearance of spontaneous arteriosclerosis. Even though the lesions

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described by these authors were found in large vessels and only non-cellular plasma constituents permeated the endothelium, their studies still support a direct toxic effect of CO on vascular endothelium. Astrup et al.¹³ further described an acceleration by CO alone in the development of atheromatosis of the aorta. They believed that the edematous condition and higher protein content of the aortic walls was due to an increased endothelial permeability. In the ultrastructural studies conducted by Kjeldsen et al.¹⁴ on the intimal changes in the rabbit aorta after moderate CO exposure, edema was evident under the basement membrane as well as the endothelial cells; often endothelium completely separated from the basement membrane and a plaque was formed. Of most interest in this study was the presence of tiny hemorrhages with platelet and red blood cell plugging in the areas of denuded endothelium. Kjeldsen et al.¹⁴ concluded that the morphologic intimal changes of the rabbit aorta were due to CO per se since the oxygen tension did not change during CO exposure. This was not the case in my experiments, since oxygen tension did decrease with a concomitant rise in COHb. Siggaard-Andersen et al.¹⁵ also reported that CO induces endothelial damage and that CO has a more pronounced effect than hypoxia alone on the permeability of capillaries to albumin. The exact mechanism by which CO increases endothelial permeability to plasma and/or cellular components of blood remains obscure; however, oxygen dependent enzymes may be necessary in order to maintain the permeability of individual endothelial cells and/or intercellular endothelial junctions. CO may in some way have a direct toxic effect upon these same enzymes.

The results of experiments on rabbit fetuses in my laboratory and those conducted by others on adult animals strongly suggest that CO can compromise the blood flow to tissues by causing a vasoconstriction of

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small arteries and veins and by increasing the endothelial permeability to plasma and/or cellular constituents of blood. These functional or morphological alterations can severely compromise the perfusion of capillaries thus impairing the proper delivery of oxygen to tissues or organs. This, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of the inactive pigment, carboxyhemoglobin.

The results from these studies lend further support to the possibility that the CO content of cigarette smoke may be the causative agent which is responsible for the lower birth weights of newborn or the higher incidence of neonatal mortality in newborns from mothers who smoke. The microvascular effects described in this study coupled with the known effects of CO on oxygenation could impair the proper delivery of blood to the growing fetus. The functional and morphologic alterations which may arise in the fetus, then, really only depends upon whether or not the microvascular response observed in the fetal mesentery is truly representative of what occurs in other microvascular beds such as the central nervous system.

The experimental protocol in this proposal will further investigate the effects of CO on the fetal microvascular system. The use of monochromatic light will provide additional information on the alterations in structure and function of the microvascular system induced by CO by enabling more critical observations of the microvascular response at one wavelength of light, for example, 414 mμ for hemoglobin. Since studies will also be conducted on the maternal microvascular response to CO, it will be interesting to ascertain if this response mimicks that in the fetus. Since several of the ultrastructural studies by Kjeldsen et al.¹⁴ and others suggest that a

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mild exposure to CO can induce pathologic changes in the walls of vessels in rabbits, it is conceivable that these changes could have an effect upon the flow of blood through these vessels. Thus, the experiments on the response of the adult microvascular system will be compared to: (1) the response of the fetus; (2) ultrastructural studies conducted by other authors in adult animals or man; and (3) the ultrastructural results of our own studies. If CO increases the permeability of endothelium to plasma constituents, then it may well be that CO does play a significant role in the development of coronary heart disease and even peripheral vascular diseases as has been suggested by several authors. If our scanning and transmission electron microscopic studies of fetal blood vessels which have been exposed to CO demonstrate a structural similarity to adult blood vessels that have been exposed to CO, then can one conclude that such morphologic changes might predispose a newborn animal (or human) to cardiovascular disease in later life? The dovetailing of information gathered from vital microscopic, scanning electron microscopic, and transmission electron microscopic studies of fetal vessels exposed to CO in this study may either support or refute this possibility.

The results from the CO experimental groups will be compared to the results from the nicotine experimental groups. Little information is available on the effects of nicotine on the fetus. The potentially harmful effects of nicotine on the fetus are just as important as those effects which may be related to CO. In fact, nicotine has been implicated more often than CO as the main harmful constituent of tobacco smoke due, in part, to its known pharmacological effects on the cardiovascular system. Nicotine is known to cross the placental barrier in some animals such as rats.¹⁶ In these animals, the fetal levels of nicotine actually exceeded the maternal levels at various intervals of time after maternal administration of radioactive nicotine. Nicotine is known to induce a significant

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increase in the amount of catecholamines released from the adrenal gland.¹⁷ If nicotine does cross the placental barrier in pregnant rabbits and if it does increase circulating levels of catecholamines due to its action on the fetal adrenal gland, then the peripheral vascular resistance and/or cardiac output might be markedly affected. Nicotine is also known to have other effects such as accelerating platelet aggregation by ADP.¹⁸ Like CO, nicotine also has been shown to affect the birth weight and neonatal or prenatal mortality rate of offspring of mothers who received nicotine.¹⁹ The vasoconstriction and breakdown of the endothelial lining of the fetal microvascular system after CO may also occur with nicotine since Matsubara and Sano²⁰ suggested that nicotine induces closure of pre-capillary sphincters in calves causing a decreased capillary filtration coefficient. Although the studies by Matsubara and Sano²⁰ were performed in calves, other authors have described the effects of nicotine on the fetus and have suggested that the response depends upon the gestational age of the fetus which, in turn, reflects the development of the autonomic nervous system and adrenal gland. Thus, nicotine can play a similar role as CO in compromising the blood flow and/or oxygenation of growing fetuses; thus it is put in a similar category as a potentially harmful etiologic agent of tobacco smoke. It will be interesting to compare the response of the fetal microvascular system to the exogenous administration of nicotine to the mother with the response of maternal carbon monoxide exposure. Then one may be able to better appreciate the mechanisms which function in the fetus to produce deleterious effects upon fetal growth and development in mothers who smoke.

As mentioned in the experimental protocol, the studies conducted on the response of the "adult" microvascular system in rabbits after exposure to CO or nicotine will be repeated in adult rats. Thus, in vivo observations

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will be conducted on the response of the mesenteric microvascular system in rats to CO or nicotine and compared to the response in "adult" rabbits. This will permit a comparison of the sensitivities to CO or nicotine of these two animals in the same microvascular bed. Furthermore, in order to study the effects of CO or nicotine on another microvascular bed, in vivo microscopic studies will be conducted in rats on the response of the hepatic microvascular system to CO or nicotine; these results will then be compared to the response in the mesentery. This will provide useful information on the sensitivities of different microvascular beds in the same animal (hepatic and mesenteric in rats) versus similar microvascular beds in two different animals (mesenteric in adult rabbits and rats).

After completion of the acute experiments outlined in this proposal, hopefully additional information will be available which either supports or refutes (a) the reported cause-and-effect relationship between cigarette smoking and fetal or neonatal development, and (b) the etiologic role cigarette smoking plays in the development of various cardiovascular diseases in adults. By examining the separate effects of CO and nicotine on the adult and fetal microvascular systems in animals, one may gain a better insight into the problem of defining what constituents of cigarette smoke are truly harmful.

C. Addendum:

In the experimental protocol, one potential experimental group was the effects of maternal smoking on the fetal or adult microvascular system. These studies depended upon the availability of a mechanical device which would simulate human exposure to cigarette smoke. I would like to re-emphasize that if such a machine becomes available, this experimental group (exposure to smoke) will be added. I believe that this would be an integral part of the proposal since one could observe in the living animal if the CO -

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response or the nicotine-response more closely followed the microvascular response of exposure to cigarette smoke.

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D. References:

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9. Physical Facilities Available :

The senior investigator has 400 square feet of laboratory space equipped with the following items.: 1 vibrationless steel optical bench for vital microscopy; quartz-rod apparatus; Leitz stereo binocular microscope modified for vital microscopy; Leitz Panphot microscope (without optics); RCA Vidicon television system; Fairchild/Dumont Vidicon television system; 8" Conrac TV monitor; Bolex H-16 Rex 5 16 mm. motion picture camera adapted for cinémicrophotography; 2-tripods; motion picture editing and storage equipment; YSI temperature control equipment; balances; Sage air curtain incubator; Heidbrink closed-circuit anesthetic machine; Wilmot Castle surgical lamp; Bausch and Lomb spectrophotometer; A. O. microtome; warming table; clinical centrifuge; A. O. microstar microscope; deionizer; microhematocrit centrifuge; IL 182 CO-oximeter.

Dr. Zimny has in her laboratory a A.E.I. - 6B transmission electron microscope; furthermore, she has available for her use a scanning electron microscope, J.S.M. - U3, at Touro Infirmary in New Orleans.

The Department of Anatomy also maintains adequate dark room and animal care facilities.

10. Additional Requirements:

None

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11. & 12. Biographical Sketches of Professional Personnel and
Pertinent Publications:

Name: Samuel G. McClugage, Jr.

Birth Date:

Birth Place: REDACTED

Education: Undergraduate: Millikin University, Decatur, Illinois
A.B. (Zoology), 1966.

Graduate: University of Cincinnati, College of
Medicine, Cincinnati, Ohio
Ph.D. (Anatomy), 1970.

Honors: N.I.H. Predoctoral Fellowship, 1967-1970

Consultant, Proctor and Gamble Company, Cincinnati, Ohio
1972 -

Recipient, Microcirculatory Society-Pharmacia Travel Award
(to visit research laboratories in Scandinavia), June, 1973.

Societies: American Association for the Advancement of Science, 1967 -
Midwestern Association of Anatomists, 1968-1971
Microcirculatory Society, 1968 -
Sigma XI, 1970 -
International Society for Experimental Hematology, 1970 -
New Orleans Section, International Association for Dental
Research, 1972 -

Research Interests: In vivo microscopy of living cells, tissues, and organs
in situ under normal or pathologic conditions; in vivo
physiologic and pharmacologic studies; microcirculation;
hematology; application of television and electronic
techniques to microscopic study of living tissues and
organs in situ.

Background: 1. Assistant Professor of Anatomy, Louisiana State
University Medical Center, 1971 - present

2. Postdoctoral Fellow in Anatomy, University of Cincinnati,
1970-1971

3. Pre-doctoral Fellow, National Institutes of Health
(GM-38179), University of Cincinnati, 1967-1970

4. Pre-doctoral Fellow, from the Dean of the College of
Medicine, University of Cincinnati, 1966 - 1967

5. Assistant Instructor in Biology, Millikin University, 1966

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Publications:

1. McCuskey, R. S., McClugage, S. G., Moore, T. J., and Miller, M. L. Response of the fetal mesenteric microvascular system to maternal hypoxia. *Proc. Soc. Exp. Biol. & Med.* 132: 636-639, 1969.
2. McCuskey, R. S., McClugage, S. G., and Younker, W. Microscopy of living bone marrow in situ. *Blood* 38: 87-95, 1971.
3. McClugage, S. G., McCuskey, R. S., and Meineke, H. A. Microscopy of living bone marrow in situ. II. Influence of the microenvironment on hemopoiesis. *Blood* 38: 96-107, 1971.
4. McClugage, S. G., and McCuskey, R. S. Relationship of the microvascular system to bone resorption and growth in situ. *Microvas. Res.* In press.
5. McClugage, S. G., and McCuskey, R. S. Microscopic study of the response of the living liver to carbon tetrachloride poisoning. *Microvas. Res.* 5: 354-360, 1971.
6. McCuskey, R. S., McClugage, S. G., and Meineke, H. A. Microscopy of living bone marrow in situ. *Experimental Hematology* 21: 33-34, 1971.
7. McClugage, S. G. Response of the fetal microvascular system to maternal carbon monoxide exposure (In preparation).

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NAME: Marilyn L. Zimny

TITLE: Professor of Anatomy

BIRTHDATE:

PLACE OF BIRTH: **REDACTED**

EDUCATION:

University of Illinois, Urbana, Illinois
Chemistry - major, Zoology - minor, B.A., 1948
Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, M.S., 1951
Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, Ph.D., 1954

PROFESSIONAL EXPERIENCE:

Professor - Anatomy, Louisiana State University Medical Center,
1964 - present
Associate Professor - Louisiana State University Medical Center,
1959 - 1964
Assistant Professor - Louisiana State University Medical Center,
1954 - 1959
Visiting Professor in Anatomy, University of Costa Rica, School
of Medicine, February-June, 1961 - 1962
Sabbatical leave, Institute of Arctic Biology, University of
Alaska, 1966
Abstractor for Biological Abstracts, 1959 to present
The World Book Encyclopedia Biology Committee

ORGANIZATIONS:

American Association of Anatomists
American Society of Zoologists
American Physiological Society
Louisiana Electron Microscope Society
Electron Microscopic Society of America
American Association for the Advancement of Science
Orthopedic Research Society

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PUBLICATIONS:

1. Zimny, M. L. and Rigamer, E. Glomerular ultrastructure in the kidney of a hibernating animal. *Anat. Rec.* 154: 87-94, 1966.
2. Zimny, M. L., Sherman, M. and Romano, C. C. Ultrastructural modifications of the intercalated disc during hypothermia in the rat and the ground squirrel. *Cryobiology* 4: 317-328, 1968.
3. Zimny, M. L. Glomerular ultrastructure in kidneys from some northern mammals. *Comp. Physiol. & Biochem.* 27: 859-864, 1968.
4. Zimny, M. L. and Redler, I. An ultrastructural study of patellar chondromalacia in humans. *Journal of Bone and Joint Surgery* 51A: 1178-1190, 1969.
5. Redler, I. and Zimny, M. L. Scanning electron microscopy of normal and abnormal articular cartilage and synovia. *Journal of Bone and Joint Surgery* 52A: 1395-1404, 1970.
6. Zimny, M. L. and Redler, I. An ultrastructural study of chondromalacia fabellae. *Clinical Orthopaedics and Related Research* 82: 37-44, 1972.
7. Zimny, M. L. and Redler, I. Scanning electron microscopy of chondrocytes. *Acta Anat.* 83: 398-402, 1972.
8. Booth, W. V., Zimny, M. L., Kaufman, H. J. and Cohn, I. Scanning electron microscopy of small bowel strangulation obstruction. *Amer. J. Surg.* 125: 129-133, 1973
9. Zimny, M. L. and Redler, I. Variations in morphology of cartilage within a given area of articular surface. (Submitted for publication, *J. Microscopy*).

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13. Budget: (1st year)

| A. Salaries (Personnel by names) | % time | Amount |
|--|--------|-------------|
| Professional | | |
| Samuel G. McClugage, Jr. | 50% | None |
| Marilyn L. Zimny | 15% | None |
| Technical | | |
| *Research Assistant (including fringe benefits) | 100% | \$ 7,920.00 |
| *Research Assistant (including fringe benefits) | 50% | 3,960.00 |
| Secretary (including fringe benefits) | 50% | 3,080.00 |
| Sub-Total | | 14,960.00 |
| B. Consumable Supplies (list by categories) | | |
| 100 Pregnant rabbits @\$20.00 | | 2,000.00 |
| 25 Non-pregnant rabbits @\$10.00 | | 250.00 |
| Anesthetic gases (CO/Air, O ₂) | | 400.00 |
| Motion Picture film and film processing | | 1,000.00 |
| Misc. supplies (chemicals, surgical instruments, etc.) | | 600.00 |
| Sub-Total | | 4,250.00 |
| C. Other Expenses (itemize) | | |
| Animal Care (.20/day/rabbit) | | 750.00 |
| *100 hours of use of Scanning Electron Microscope @\$20.00/hr. | | 2,000.00 |
| Travel (for two people to attend one meeting per year) | | 600.00 |
| *Machinist expenses | | 400.00 |
| Sub-Total | | 3,750.00 |
| D. Permanent Equipment (itemize) | | |
| *Monochromatic system adapted for quartz rod | | 2,150.00 |
| *Optical equipment necessary to adapt Leitz Panphot Microscope for <i>in vivo</i> microscopy | | 4,790.00 |
| *Low light level Cohu television camera including sync. generator | | 4,750.00 |
| Sub-total | | 11,690.00 |
| E. Overhead (15% of A + B + C) | | |
| Overhead | | 3,444.00 |
| Total | | 38,094.00 |

Estimated Future Requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Overhead | Total |
|--------|-----------|-------------------|----------------|------------------|----------|-----------|
| Year 2 | 15,758.00 | 4,250.00 | 3,350.00 | 1,400.00 | 3,504.00 | 28,262.00 |
| Year 3 | 16,405.00 | 4,250.00 | 3,350.00 | 900.00 | 3,601.00 | 28,506.00 |

Salaries include increments of 6% per year plus 10% for fringe benefits

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

* (See Justification of Budget on next page)

Signature Samuel G. McClugage, Jr.
 Director of Project Samuel G. McClugage, Jr.
 (504) 947-9961 - ext. 255 Telephone
 Signature _____
 Business Officer of the Institution E. F. Pohlrig
 Comptroller (504) 527-5142 Telephone

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13. Budget: (1st year)

| A. Salaries (Personnel by names) | | % time | Amount |
|--|------|----------|-------------|
| Professional | | | |
| Samuel G. McClugage, Jr. | | 50% | None |
| Marilyn L. Zimny | | 15% | None |
| Technical | | | |
| *Research Assistant (including fringe benefits) | 100% | | \$ 7,920.00 |
| *Research Assistant (including fringe benefits) | 50% | | 3,960.00 |
| Secretary (including fringe benefits) | 50% | | 3,080.00 |
| Sub-Total | | | 14,960.00 |
| B. Consumable Supplies (list by categories) | | | |
| 100 Pregnant rabbits @\$20.00 | | | 2,000.00 |
| 25 Non-pregnant rabbits @\$10.00 | | | 250.00 |
| Anesthetic gases (CO/Air, O ₂) | | | 400.00 |
| Motion Picture film and film processing | | | 1,000.00 |
| Misc. supplies (chemicals, surgical instruments, etc.) | | | 600.00 |
| Sub-Total | | | 4,250.00 |
| C. Other Expenses (itemize) | | | |
| Animal Care (.20/day/rabbit) | | | 750.00 |
| *100 hours of use of Scanning Electron Microscope @\$20.00/hr. | | | 2,000.00 |
| Travel (for two people to attend one meeting per year) | | | 600.00 |
| *Machinist expenses | | | 400.00 |
| Sub-Total | | | 3,750.00 |
| D. Permanent Equipment (itemize) | | | |
| *Monochromatic system adapted for quartz rod | | | 2,150.00 |
| *Optical equipment necessary to adapt Leitz Panphot Microscope for <u>in vivo</u> microscopy | | | 4,790.00 |
| *Low light level Cohu television camera including sync. generator | | | 4,750.00 |
| Sub-total | | | 11,690.00 |
| E. Overhead (15% of A+B+C) | | Overhead | 3,444.00 |
| | | Total | 38,094.00 |

Estimated Future Requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Overhead | Total |
|--------|-----------|-------------------|----------------|------------------|----------|-----------|
| Year 2 | 15,758.00 | 4,250.00 | 3,350.00 | 1,400.00 | 3,504.00 | 28,262.00 |
| Year 3 | 16,405.00 | 4,250.00 | 3,350.00 | 900.00 | 3,601.00 | 28,506.00 |

Salaries include increments of 6% per year plus 10% for fringe benefits

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Concerning Conditions and Terms Under Which Project Grants Are Made."

* (See Justification of Budget on next page)

Signature Samuel G. McClugage, Jr.
 Director of Project Samuel G. McClugage, Jr.
 (504) 947-9961 - ext. 255 Telephone
 Signature E. F. Pohlig
 Business Officer of the Institution E. F. Pohlig
 Comptroller (504) 527-5142 Telephone

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13. Justification of Budget:

A. Personnel

The personnel that will be required on this project are one full-time research assistant for Dr. McClugage and a half-time research assistant who will work with Dr. Zimny in the preparation of tissues for scanning and transmission electron microscopy. The percent used for calculating fringe benefits at Louisiana State University is 10% which has been included in the "amount column" of the budget.

B. Use of Scanning Electron Microscopy:

The Department of Anatomy at Louisiana State University Medical Center does not have a scanning electron microscope (SEM). However, we have an agreement with the Research Institute at Touro Infirmary in New Orleans to rent their SEM at a rate of \$20.00/hour. Dr. Zimny has access to this microscope whenever its use is needed.

C. Machinist Expenses:

The employment of a machinist who can make the necessary animal trays for use on the Paraphot microscope, adapted for vital microscopy, will be necessary. These trays must meet certain specifications depending on the type of animal used and the particular organ which is to be observed in vivo.

D. Permanent Equipment :

1. Monochromatic System Adapted for Quartz Rod

Schoeffel Instrument Corporation recently manufactured a monochromatic system which provides maximum light energy (from 200-700nm.) with high spectral purity. The complete system consists of a Xenon or Xenon-Mercury arc lamp, power supply, arc lamp housing, double monochromators, and appropriate optics. The double monochromators provide a narrow spectral

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bandwidth at the selected wavelengths while suppressing stray light at other wavelengths to 1 part in 100,000. This system has a focusing sleeve at the exit portal which would permit the beam being focused on the quartz rod which has been used to date for transillumination using only white light. The main problem with white light is the inability to selectively build-up the contrast of the optical system. The use of monochromatic light permits the selection of wavelengths of light that are absorbed by specific tissue and cellular components. This differential absorption of light by these structures enhances their contrast with the surrounding structures and aids in their visual recognition. When such differences of absorption are sensed by the television tube and converted into an electronic image, the contrast between tissue and cellular components can be enhanced further by adjustment of the brightness and contrast controls on the video monitor. For example, patterns of blood flow can be followed more easily than by using white light by selecting a wavelength of light that is absorbed maximally by hemoglobin in red blood cells (414 mμ). This system will allow more critical observations of the linear velocity of blood flow through the microvascular system as well as passage of these cells through the endothelium of these vessels.

2. Optical Equipment for Panphot Microscope:

At the present time in my laboratory, a fused quartz rod is being used as a light source. The use of a quartz rod (coupled with a monochromatic system) as a transilluminatory light source can provide an adequate amount of light for transillumination of thin tissues such as the fetal or adult mesentery in the rabbit. However, the investigator is somewhat limited in respect to the tissues or organs selected for study since relatively low magnifications are used. Thicker tissues or organs require higher intensities of light in order to

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transilluminate through them. This entails the use of a condenser in the optical system. I have in my laboratory a used Leitz Panphot binocular microscope, without optics. This microscope can be adapted for vital microscopy which then can be used for direct in vivo observations of tissues or organs using transmitted or reflected light or alternatively, television microscopy as mentioned in one above. Thus, the part of the experimental protocol which requires examination of organs such as liver in rats can be accomplished. (I have included a reprint, Microvas. Res. 3: 354-360, 1971, which will illustrate the methodology used for television microscopy and how it can be used to study living organs and tissues in situ).

3. Low Light Level Television Camera:

As mentioned in two above, when transilluminating through thick tissues or organs using either a quartz rod or a focusing condenser on a microscope, the amount of light passing through the specimen is greatly reduced from that which would pass through a 10 μ thick histologic slide. Thus, the conservation of light becomes imperative. To help offset this loss of light, a higher intensity light source can be used in conjunction with a television system which can provide useful pictures under compromised lighting conditions. The low light level Cohu television camera (2850 series) containing a silicon diode-array vidicon can be used in such conditions. The automatic light range controls are fully operational for scene brightness changes from 0.5 footlambert to 25,000 footlamberts with an f1.4 lens. After seeing a demonstration of this camera, I am convinced of its applicability to television microscopy under compromised lighting conditions and of its superiority over the two vidicon cameras I presently have in my laboratory.

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

| Title of Project | Source | Amount | Duration |
|--|-----------------------------|----------|-----------------------------------|
| Response of the fetal mesenteric microvascular system to maternal carbon monoxide exposure | Louisiana Heart Association | 7,300.00 | July, 1972- June, 1973 |
| <u>In vivo</u> model for testing effects of pulp capping agents on dental pulp | Institutional Grant | 900.00 | March, 1973- February, 1974 |

Pending

(None)

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